

1988

Thymic structure and function in aging dogs

Belinda Lawler Goff
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Thymic structure and function in aging dogs

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Iowa State University, 1988

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Ann Arbor, MI 48106**

Thymic structure and function
in aging dogs

by

Belinda Lawler Goff

A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of the
Requirements for the Degree of

DOCTOR OF PHILOSOPHY

Interdepartmental program: Immunobiology

Major: Immunobiology

Approved:

Signature was redacted for privacy.

In Charge of Major Work

Signature was redacted for privacy.

Chair, Immunobiology Program

Signature was redacted for privacy.

For the Graduate College

Iowa State University
Ames, Iowa
1988

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GENERAL INTRODUCTION

Advancing age is associated with involution of the thymus, decreased thymic endocrine function, and depressed immune function (Delafuente, 1985; Schuurman et al., 1985). Increased incidence of certain 'diseases of aging' are also noted (Schultz, 1984; Monroe and Roth, 1986). An agent or agents that would stimulate the immune system in the aged might also be associated with better health as assessed clinically.

Various hormones have been observed to enhance thymic morphology and immune function. A review of the literature regarding various thymotrophic agents is presented in Section I. One such thymotrophic agent is growth hormone (GH). The results of a study on the effects of bovine GH on thymic structure and endocrine function in aging dogs is presented in Section II.

Since exogenous GH may be recognized as a foreign protein by dogs, long term administration may lead to the production of antibodies against it, negating its beneficial effects. Previous results in our laboratory indicate that oral clonidine HCl stimulates the release of endogenous GH in aging dogs. However, daily administration was associated with a loss of the GH response to clonidine. The effect of intermittent administration of oral clonidine on GH release, thymic structure and immune function in aging dogs is presented in Section III.

The final part of the research for this dissertation involved the study of thymic cysts in the age-involuting canine thymus (Section IV). Similar structures have been reported in other species and have been hypothesized to have a secretory function. This study provides the first

direct evidence of the presence of at least two thymic hormones within the cyst epithelial cells. The light microscopic anatomy and histochemical analysis of canine thymic cysts have been previously reported and were reconfirmed here. The electron microscopic appearance of these cysts in the dog has not been previously reported. The ultrastructure of the cyst epithelium is described and compared to that reported in other species.

EXPLANATION OF THE DISSERTATION FORMAT

An alternative format was used in this dissertation. There are four sections after the General Introduction; each of the sections is an individual paper. The first section is a review of the literature which will be submitted for publication, the second section has been published in Clinical and Experimental Immunology, the third section will be combined with results of an ongoing experiment and then will be submitted for publication, the fourth section will be submitted for publication. Finally, a general summary and discussion of the dissertation is included.

SECTION I: THYMOTROPIC AGENTS

THYMOIROTIC AGENTS

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INTRODUCTION

The thymus gland is an organ located in the anterior mediastinum, and it plays an integral role in development and maintenance of cellular immunity (Symmers, 1966; Weksler, 1982). The thymus gland from a young animal is characterized histologically by an abundance of lymphocytes, especially in the cortical region, a distinct corticomedullary junction, and the absence of adipose tissue either in the thin interlobular septae or in the parenchyma (Figure 1a). Age-related involution of the thymus, which begins at about the time of puberty, is associated with massive loss of lymphocytes, especially from the cortex (Figure 1b). This lymphocyte loss leads to an indistinct corticomedullary junction and a decrease in the cortex to medullary ratio. Interlobular septae become wider as thymic lobules reduce in size and separate from each other. The amount of adipose tissue progressively increases within the septae and parenchyma. In some species involution is accompanied by the apparent hypertrophy of the thymic epithelium, with some forming cysts of a secretory nature (Goff, 1988). Concurrent with thymic morphological involution is a decline in thymic endocrine function, as assessed by the waning secretion of various thymic hormones (Goldstein et al., 1974; Bach et al., 1975; Lewis et al., 1978).

In general, the normal function of the immune system declines with age, with T-cell mediated immunity being especially affected (Hirokawa and Makinodan, 1975; Weksler et al., 1978; Delafuente, 1985). Although decreased, the immune function of the thymus continues to be important throughout adult life. Neonatal thymectomy rapidly leads to lymphoid

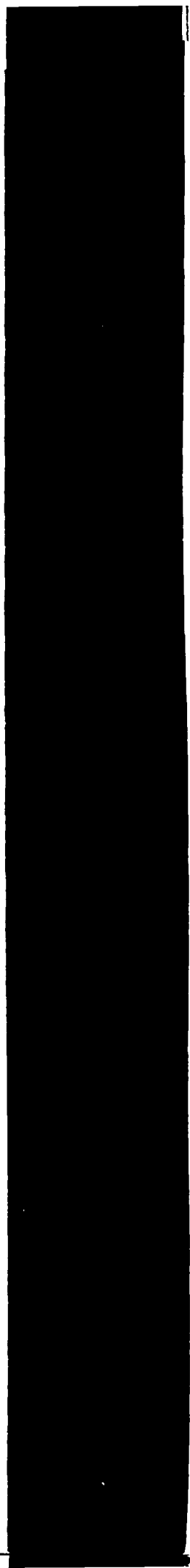
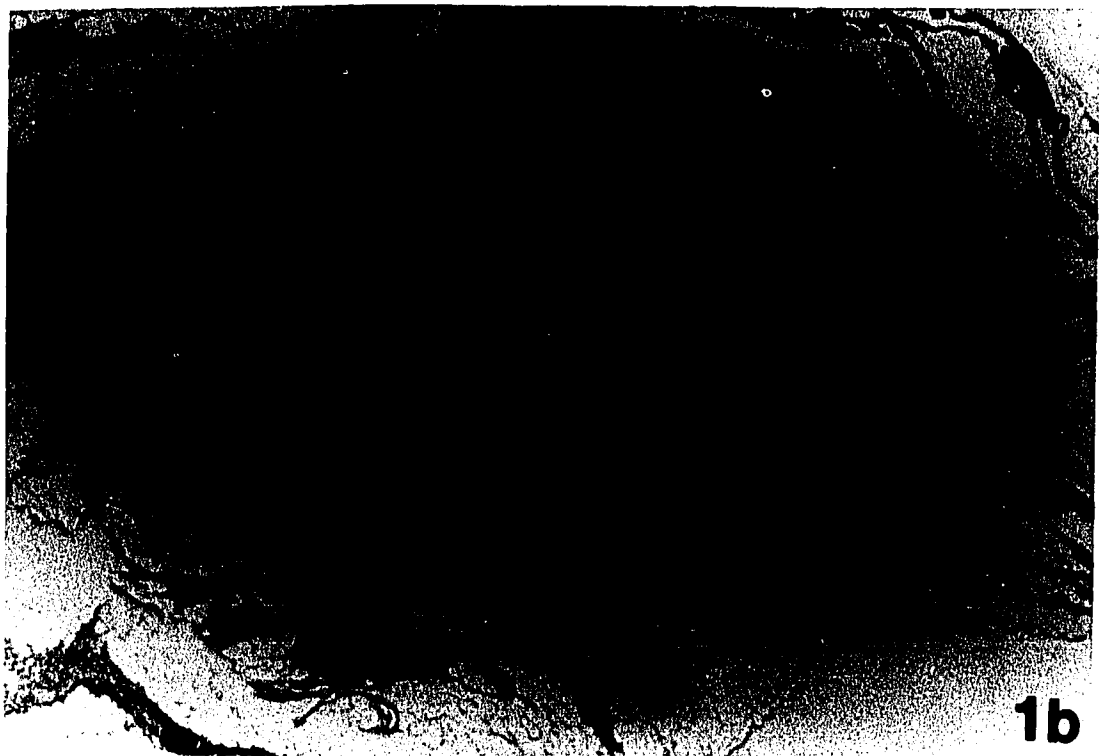


Figure 1. Photomicrographs of thymus glands from young (a) and old (b) dogs. (bar 500um)

a, Thymus from a dog approximately 4 months old. Note the distinct corticomedullary junction (j), thin interlobular septae (s), and the absence of adipose tissue. The cortex (c) to medullary (m) ratio is 1:1 or greater and there are numerous lymphocytes present in the cortex.

b, Thymus from a dog 7 years of age. The corticomedullary junction is indistinct and the cortex to medullary ratio is less than 1:1 with a depletion of cortical lymphocytes. The bracket (j) demarcates the remnants of a thymic lobule. The amount of adipose tissue (a) present in the parenchyma and interlobular septae increases with age as does the incidence of thymic cysts (c)



organ atrophy, generalized immunological deficiencies, and a progressive wasting disease leading to early death (Metcalf, 1965; Miller, 1965; Taylor, 1965). The effects of adult thymectomy are delayed since an adequate pool of competent cells remain. However, eventually a striking depletion of cells in the secondary lymphoid organs and a defective immune response to newly encountered antigens becomes apparent (Metcalf, 1965; Miller, 1965; Taylor, 1965). Thymic atrophy may also occur precociously in the face of physiological imbalance, stress, or disease.

The changes in thymic morphology and in some parameters of immune function that occur with age and in precocious involution are reversible. Several factors have been observed to regenerate or enhance thymus structure and immune function. The effects of these thymotrophic factors on thymus structure and immune function are the topic of this review.

PITUITARY - THYMUS INTERACTIONS

The thymotrophic effect of the pituitary hormones growth hormone (GH) and prolactin (PRL) has been frequently reported. There is some sequence homology between these two adenohypophyseal hormones, and pituitary derived preparations of one may be contaminated to some extent with the other. Still, a preponderance of the evidence (as follows) indicates that GH has a more important role in affecting thymic morphology than does PRL.

Kelley et al. (1986, 1987) have observed thymic regeneration and enhancement of some parameters of immune function in aging female Wistar-Furth rats implanted with GH₃ pituitary adenomas. The GH₃ tumor secretes both GH and PRL at a ratio of approximately 70:30; GH₃ implanted rats have a 20 to 100 times greater GH concentration and a 10 times greater PRL concentration in their plasma (Boockfor et al., 1985; Kelley et al., 1987). Two months after GH₃ tumor implantation, thymic morphology similar to 3 month old controls was observed in 18 month old rats, while 24 month old rats showed only partial thymic regeneration (Kelley et al., 1986). Similarly, the splenic lymphocyte response to the phytoimitogens phytohemagglutinin (PHA) and Concanavalin A (Con A) was restored to values comparable to 3 month old controls in GH₃ implanted 18 month old rats (Kelley et al., 1986). In GH₃ implanted 24 month old rats the mitogenic response was significantly increased as compared to age matched controls, but remained about 90% less than the response observed in the young controls; Interleukin-2 (IL-2) synthesis from Con A-activated splenocytes was increased from 2% to 50% of young control values (Kelley et al., 1986). Thymic tissue from the 24 month old GH₃-implanted rats had a

moderately higher proportion of lymphocytes with the Thy-1.1 and T_H (helper) phenotypes as detected by flow microfluorimetry (Kelley et al., 1986). It was concluded that GH and/or PRL from the GH_3 pituitary adenomas augmented thymic size and reconstituted mitogenic responses in aged rats (Kelley et al., 1986).

In attempting to sort out the GH from the PRL effect on thymic morphology and immune function Davila et al. (1987) administered ovine GH (oGH) to 26 month old, female Fischer 344 (F344) rats, twice daily for five weeks. The oGH treatment was associated with enhancement of splenocyte proliferation to mitogens and natural killer cell (NK) activity at low target to effector ratios. Thymic morphology at necropsy and IL-2 synthesis were not significantly different from age-matched, saline-injected controls; however, thymic morphology in the control rats did not show the expected signs of age-associated atrophy that would negate any thymotrophic effect of GH when structural comparisons were made. It was concluded that PRL, in addition to GH (as from GH_3 cells), may be necessary for enhanced thymic structure and some parameters of immune function in aging rats; undefined factors secreted by GH_3 pituitary cells, the discontinuous (daily) injection regimen and the stress associated with it may have been confounding factors affecting the results of these studies. To overcome some of these problems, an experimental design in which groups of rats received either GH, PRL, both GH and PRL, GH_3 tumors, or saline all within the framework of a single experiment could be attempted.

Growth hormone treatment has been associated with enhanced thymic morphology and function in the aging dog. Monroe et al., (1987)

administered bovine GH (bGH) to five dogs between the ages of approximately 2.5 - 5 years, with five additional dogs serving as controls and receiving bovine serum albumin (BSA). The regimen of bGH injection in this study was 0.1 mg/kg daily for five doses, then every other day for five doses, then every third day for four doses for a total of 14 doses in 27 days. Thoracotomy 10 days to three weeks before bGH treatment was begun revealed moderate to severely atrophied thymic morphology in all dogs. At necropsy, all five bGH treated dogs had regenerated thymic morphology. Control dogs, treated with BSA, had not regenerated as consistently or as markedly as bGH treated dogs although two of the five controls did have regenerated thymus glands. The total peripheral white blood cell count, lymphocyte count, and serum concentration of thymosin alpha 1 did not change with bGH treatment; the response of peripheral lymphocytes to mitogens did not change with treatment, though results were difficult to interpret due to assay variability and variation in response between dogs. Considering the great variability in lymphocyte function and the possible confounding factors of unknown age and medical history, the surgical biopsy procedure and accompanying anesthesia, Monroe et al., (1987) could only conclude that bGH treatment contributed to thymic regeneration in these aging dogs, but may not have been the only factor involved.

In a similar, but more controlled, study using retired breeder, female beagle dogs of known age and medical history, Goff et al. (1987) found enhanced thymic morphology and function after bGH treatment. Pituitary-derived bGH was administered as described above (Monroe et al., 1987) to middle-aged (33 - 55 months) and old-aged (63 - 83 months) dogs.

Pre-treatment thymic biopsies were not performed. Stereological assessment of thymic morphology revealed regeneration in four of five bGH-treated middle-aged dogs and in one of five BSA-treated controls at necropsy; no improvement in thymus morphology was observed in any of the old-aged dogs in this study. More frequent administration of bGH to a group of old-aged dogs was not associated with improvement of thymic morphology. However, all bGH-treated dogs, regardless of age or the state of thymus morphology at necropsy, had significantly elevated plasma concentrations of thymulin as compared to the BSA-treated, age-matched controls; the elevated thymulin concentrations were comparable to those of 4 month old dogs. It was concluded that exogenous GH may be useful for restoration of thymus structure and some immune functions in aging individuals (Goff et al., 1987). It is important to note that immunoenhancement (as measured by thymus endocrine function) did not require regeneration of thymus morphology.

Some of the earliest experiments indicating the relationship between pituitary hormones, thymus structure and immune function were performed on mutant mice. The Snell-Bagg and Ames pituitary dwarf mouse strains have a shortened life span and are deficient in GH, PRL, thyroid stimulating hormone (TSH), and adrenocorticotrophic hormone (ACTH), and develop a wasting syndrome (Fabris et al., 1971; Monroe and Roth, 1986). Concurrent with hypopituitary function is a deficient immune system, especially cell-mediated immunity, including marked thymic atrophy beginning at 15 days of age, and a decreased plasma thymulin concentration (Monroe and Roth, 1986; Fabris et al., 1971). Growth hormone therapy alone, or in combination with thyroxine, was associated with reconstitution of thymus morphology,

improved antibody response to thymus-dependent antigens, and enhanced thymulin production (van Buul-Offers and Van den Brande, 1981; Pierpaoli et al., 1969; Baroni et al., 1969). If the dwarf mice were thymectomized before GH and/or thyroxine therapy the effects of immune system restoration, prolonged life span, and reversal of the wasting disease did not occur (Fabris et al., 1971; Fabris et al., 1972; Sorkin et al., 1972). These results uphold the relationship between pituitary function, thymus structure and immune function and implicate their malfunction as a cause of the immunodeficiency and shortened life span in dwarf mice.

Immunodeficient dwarfism in an inbred colony of 7/8 Weimaraner-1/8 German Shepherd dogs was associated with a wasting syndrome at weaning, severe thymic hypoplasia, decreased peripheral lymphocyte response to PHA, and significant depression of GH release in response to clonidine (Roth et al., 1980). Treatment with bGH resulted in clinical improvement and regeneration of thymic morphology as compared to pretreatment biopsies, but was not associated with a significant increase in lymphocyte blastogenesis (which was highly variable) or thymosin alpha 1 concentrations in plasma (Roth et al., 1984). The results of studies in dwarf dogs and mice indicate the relationship between pituitary hypofunction and T-cell dependent immunodeficiencies and also demonstrate the role of GH in improving some of these deficits, including thymic morphology.

Prolonged nursing or intraperitoneal injections of mouse milk in dwarf mice prevented the development of thymic atrophy and depressed immune function associated with these mutants (Duquesnoy and Good, 1970;

Duquesnoy, 1971). The immunoenhancing agent(s) in milk are not known, but may include GH and PRL (Duquesnoy, 1971).

The presence of receptors for GH on the membranes of thymocytes has been reported (Talwar et al., 1974; Pandian et al., 1975). Hanjan and Talwar (1975) studied the in vitro effect of GH on surface charge and electrophoretic mobility of thymocytes from aged rats. The percentage of GH-sensitive thymocytes decreased from 65% to 19% by 1 year of age, and no effect was detectable in 1 1/2 year old rats; those thymocytes still responding to GH in the older rats studied did so to the same extent as young rat thymocytes (Hanjan and Talwar, 1975). It was concluded that it is not the density and quantitative disposition of receptors for GH that diminish with age, rather the number of cells reacting with the hormone decreases (Hanjan and Talwar, 1975).

Hypophysectomy (the surgical removal of the pituitary gland) results in thymic atrophy resembling that in age-involution or the naturally occurring dwarf mutants. Comsa et al. (1979) hypophysectomized male Sprague-Dawley rats at 35 days of age, then injected them with GH daily for 15 days. Rats treated with GH had thymus morphology apparently restored to normal in contrast to saline-injected controls at necropsy (Comsa et al., 1979). Nagy and Berczi (1978) observed restoration of the suppressed immune response in hypophysectomized rats after PRL administration.

Another experimental approach in the study of pituitary-immune interactions is the use of anti-hypophysis or anti-GH antiserum. Thymic atrophy and a wasting syndrome resulted when rabbit anti-mouse hypophysis serum was administered to young mice at an age when thymectomy would cause

a wasting syndrome (Pierpaoli and Sorkin, 1967). Similar results were observed when anti-bGH serum was administered to young mice but the effect could be blocked by simultaneous GH treatment (Pierpaoli and Sorkin, 1968). The effect of GH in blocking the development of the wasting syndrome is apparently via its action on the thymus since its effect was negated in neonatally thymectomized mice (Fabris et al., 1970).

Reciprocally, thymectomy has been associated with changes in adeno-hypophyseal acidophils (sites of GH and PRL production). Pierpaoli et al. (1971) observed enlarged and degranulated adeno-hypophyseal cells, with endoplasmic reticulum enlarged to cisterns, in thymectomized mice. These are expected occurrences in endocrine cells which have lost the feedback influence from a target organ.

THYROID - THYMUS INTERACTIONS

Thyroid hormones have been observed to have a thymotropic action when administered to various thyroid deficient animals, and may synergize with other hormones (especially GH) to this end.

Few studies have directly addressed the question of thyroid hormones as thymotropic factors in aging animals. Fabris et al., (1982a) observed that the plasma concentrations of triiodothyronine (T_3) and thyroxine (T_4) in Balb/c mice remained relatively constant from 3 to 12 months of age; beyond this age T_4 concentrations progressively decreased while T_3 concentrations increased until 20 months of age and declined thereafter. Treatment of aging, male Balb/c mice with L-thyroxine reversed the age-dependent decline of various parameters of immune function including: plaque-forming-cell capacity (after immunization with sheep red blood cells [sRBC's]), splenocyte response to PHA, and plasma concentration of thymulin (Fabris et al., 1982a). No significant enlargement of lymphoid organs was noted, although the data were not presented (Fabris et al., 1982a). Similar enhancement of thymulin concentration and lymphocyte response to PHA was observed in aging Balb/c mice that received a neonatal thymus graft (Fabris et al., 1982b). From these results it was concluded that thyroxine acts at the level of the thymus to exert effects on immune function in aging mice (Fabris et al., 1982b).

Hyper- and hypothyroidism in animals and humans is observed to be associated with hypertrophy or involution of the thymus, respectively (Fabris et al., 1987; Shivatcheva and Hadjioloff, 1987). Hyperthyroidism

is also associated with increased plasma concentrations of thymulin (Fabris et al., 1986).

Pituitary dwarf mice, as discussed in the previous section, have immune function deficits associated with decreased concentrations of pituitary hormones including thyroid stimulating hormone (TSH), GH, PRL, and ACTH, and have shortened life spans (Pierpaoli et al., 1969). Administration of GH alone or with thyroxine was observed to improve thymic morphology in these mice (Van Buul-Offers and Van den Brande, 1981; Pierpaoli et al., 1969).

The sex-linked dwarf chicken has normal plasma GH concentrations and near normal thyroid activity, but peripheral conversion of T_4 to T_3 is decreased resulting in a functional hypothyroid condition (Scanes et al., 1982; Marsh et al., 1984). Thyroxine administration significantly stimulated thymus growth in dwarf chickens, while GH and thyroxine acted synergistically resulting in an even greater thymotropic effect (Marsh et al., 1984). Interestingly, GH and GH-thyroxine had a bursotrophic effect in dwarf chickens; GH, but not thyroxine or GH-thyroxine, was associated with an increased antibody response to sRBC's (Marsh et al., 1984).

Thyroidectomized or propylthiouracil-treated rats have thymic morphology similar to that associated with age-involution, as well as decreased plasma concentrations of thymulin (Comsa et al., 1979; Savino et al., 1984). Thymic morphology was reconstituted by thyroxine administration in thyroidectomized rats (Comsa et al., 1979).

Seasonal involution and hypertrophy of thymus morphology and immune function have been reported in several species including the frog (Plytycz and Bigaj, 1983), ground squirrel (Shivatcheva and Hadjioloff, 1987),

chicken, and a variety of wild birds such as mallards, robins, and house sparrows (Glick, 1984). In frogs and squirrels, thymic involution was observed to begin before hibernation with subsequent thymic hypertrophy occurring after the hibernation period during the return to active life (Plytycz and Bigaj, 1983; Shivatcheva and Hadjioloff, 1987). In wild birds, peak thymic development was observed to follow the annual breeding season and was hypothesized to be related to increased thyroid activity associated with molting (Hohn, 1956; Glick, 1984).

GONAD - THYMUS INTERACTIONS

The interactions between the gonadal steroids and the immune system have been reviewed recently (Grossman, 1985). In general, the gonadal steroids are inhibitory to cell mediated immune responses. Castration before the time of puberty delayed the onset of thymic involution, producing thymic hypertrophy instead (Castro, 1974). In rats, castration after puberty, and even much later, was associated with thymic regeneration and enhanced immune response to antigen (Greenstein et al., 1987).

Chemical castration by the administration of a potent analogue of the hypothalamic luteinizing hormone releasing hormone (LHRH) resulted in reversible inhibition of testicular steroidogenesis and spermatogenesis (Linde et al., 1981) and was associated with thymic regeneration in old male rats (Greenstein et al., 1987). The mechanism of action of the LHRH analogue was hypothesized to be via desensitization (negative feedback on the pituitary, resulting in decreased LH release) although a direct action on the thymus was not ruled out (Greenstein et al., 1987).

Seasonal fluctuation in the morphology of the avian thymus was attributed in part (along with thyroid function) to seasonal regression of the testes and androgen production (Glick, 1984).

In addition to the thymotrophic effect, castration in males and females of many species has also been associated with an increased rate of graft rejection (Graff et al., 1966), enhanced thymocyte response to mitogens (Grossman and Roselle, 1983), and increased peripheral white

blood cell counts (Greenstein et al., 1987). These results indicate that immune function as well as thymic morphology is affected by gonadectomy.

The thymotrophic effect of castration is counteracted by the administration of gonadal steroids (Comsa et al., 1979; Fitzpatrick and Greenstein, 1987). In orchidectomized rats treated with estradiol or testosterone, the thymus glands weighed 50% less than controls and were histologically involuted; total white blood cell counts were also decreased, reflecting primarily lymphocyte loss (Fitzpatrick and Greenstein, 1987). Progesterone treatment was associated with decreased thymic weight but did not affect total white blood cell counts (Fitzpatrick and Greenstein, 1987).

Pregnancy in mice was associated with thymic involution, especially of the steroid sensitive cortical lymphocytes; the steroid resistant medullary lymphocytes were not affected (Grossman, 1985).

Steroid receptors for estrogen, androgen and progesterone have been identified and characterized in thymic tissue (Grossman, 1985). Most authors have reported that the gonadal steroid receptors are in the epithelial reticular cells of the thymus and not in the thymocytes themselves; however, two authors have reported the presence of androgen and estrogen receptors in T-lymphocytes (Grossman, 1985), though the following experiments tend to refute these results.

Grossman et al. (1982) and Grossman and Roselle (1983) performed a series of experiments regarding the mode of action of estradiol on the cell mediated immune response. When added to the culture medium of a lymphocyte blastogenesis assay, serum from castrated rats was associated with a significant increase in the response to the mitogens PHA and Con A,

as compared to serum from normal controls. Serum from rats that had been thymectomized or castrated and thymectomized was associated with significantly lower responses to the mitogens, as compared to that from castrated rats. Physiological concentrations of estradiol or testosterone added to the medium containing normal rat serum had no effect on the response to mitogens, as compared to normal rat serum alone. However, serum that was obtained from castrated rats that had been treated in vivo with physiological concentrations of estradiol significantly decreased the mitogenic response as compared to castrate serum. It was concluded that the release of a thymic serum factor was inhibited in vivo in the presence of estradiol, and was stimulated in the absence of estradiol (serum from castrate male). These results indicate the need for consideration of hormone effects not only on lymphocytes themselves, but also on the thymic epithelial cells that affect their differentiation and function.

The action of estrogens and androgens is primarily noted in suppression of cell mediated immunity, but they may affect different cell types. It was hypothesized that estrogen enhances the antibody response due to its inhibitory effects on T_S (suppressor) cells (discussed later), while androgens may affect the antibody response through their metabolic conversion to estrogens (Grossman, 1985). The net stimulatory or inhibitory effect on the immune response may be dependent on the ratio of estrogens to androgens (Grossman, 1985).

Subpopulations of mouse lymphocytes may vary in their ability to be modified by sex steroids. For example, estradiol treatment in mice decreased the percentage and absolute numbers of cells identified as immature thymocytes and T_S precursors but did not affect the percentage of

cells identified as mature thymocytes and T_H (helper) precursors (Novotny et al., 1983). These results are consistent with the observation of increased frequency of autoimmune disease in females, including systemic lupus erythematosus, idiopathic thrombocytopenic purpura, and rheumatoid arthritis (Grossman, 1985).

ADRENAL - THYMUS INTERACTIONS

The influence of the pituitary-adrenal axis on the immune system has recently been reviewed (Berczi, 1986a). There is a complex relationship between the adrenal corticosteroids and thymus morphology and immune function. In comparing the results of experiments addressing adrenal-immune interactions one must consider: the dosage of glucocorticoids administered (physiological versus pharmacological); whether a steroid sensitive (mouse, rat, hamster, and rabbit) or steroid resistant (guinea pig, monkey, human) species was used; and, whether cortisone sensitive or resistant cell populations were being assayed. The effect of other stressors, such as handling, must also be taken into consideration since they can cause glucocorticoid release, thereby confounding the results. Thus, the vast literature on this topic does contain some apparent contradictions. In general, adrenal hypofunction or adrenalectomy results in thymic hypertrophy while adrenal hyperfunction, exogenous adrenal corticosteroids, and 'stress' are associated with thymic atrophy and immunosuppression (Berczi, 1986a).

Thymic hypertrophy was observed following adrenalectomy; normal (non-hypertrophied) thymic morphology returned in adrenalectomized rats receiving implants of corticosterone and aldosterone while desoxycorticosterone treatment was associated with some degree of hyperplasia (Comsa et al., 1979). Adrenal corticosteroid treatments associated with enhanced thymic morphology also increased the secretion of thymic hormone, measured as crude extract (Comsa et al., 1979). These

results indicate some variability of effect of various adrenal hormones on thymic morphology.

Adrenocorticotrophic hormone (ACTH) was associated with increased adenine uptake in the whole thymus, and increased oxygen consumption and thymidine uptake by thymic epithelial cells (Comsa et al., 1979). These results indicate that ACTH itself can enhance thymic function. This contradicts the previous observations that ACTH antagonized the effects of GH and PRL on the immune system (review: Berczi, 1986a).

Fitzpatrick et al. (1985) reported that the presence of the adrenals did not prevent regeneration of thymic morphology in old rats that had been orchidectomized. It was concluded that the adrenal cortex does not have as important a physiological role as the testes in age-related atrophy of the thymus (Fitzpatrick et al., 1985).

The effect of exogenous glucocorticoids on thymic morphology in rats and humans has been observed to occur quickly, within 48 hours (Weaver, 1955; Bloodworth et al., 1975). Tyan (1979) has observed that susceptibility to the effects of hydrocortisone on thymus atrophy in mice is associated with the major histocompatibility (MHC) antigens; mice having H-2^a MHC antigens were more sensitive to hydrocortisone-induced thymic atrophy than mice of other MHC types.

Cortisone sensitive thymocytes are located primarily in the thymic cortex whereas medullary thymocytes are more cortisone resistant (Weissman, 1973), which accounts for the massive loss of predominantly cortical lymphocytes in the stress-atrophied thymus. This loss of cortical lymphocytes results in the decreased cortex to medullary ratio observed in the stress-atrophied thymus. Their in vivo resistance to

glucocorticoids is lost when medullary thymocytes are tested in vitro (Weissman and Levy, 1975; Berczi, 1986a). It has been hypothesized that thymic humoral factor, produced by the thymus, confers cortisone resistance to the thymocytes (Trainin et al., 1974); pharmacological doses of glucocorticoids have a detrimental effect on thymic epithelial cell secretion (Berczi, 1986a).

The organs of the immune system, including the thymus, are innervated by the autonomic nervous system; lymphocytes and other cells in these organs are thus exposed to the catecholamine, norepinephrine (Felten et al., 1985). The major circulatory catecholamine, epinephrine, is produced almost exclusively by the adrenal medulla; norepinephrine is released by sympathetic nerve endings and to a lesser extent by the adrenal medulla (Berczi, 1986a). Catecholamine synthesis is regulated by nerve stimulation and by ACTH mediation of glucocorticoid synthesis; glucocorticoids control the rate limiting enzyme of catecholamine synthesis, tyrosine hydroxylase (Berczi, 1986a). Catecholamine synthesis increases greatly in response to stress (Berczi, 1986a). Epinephrine was observed to have a synergistic effect with corticotropin releasing factor (a hypothalamic hormone) in stimulating the release of ACTH from the pituitary (Labrie et al., 1984). Catecholamines had an inhibitory effect on the release of other pituitary hormones, such as PRL and GH (Huang and McCann, 1983; Labrie et al., 1984). Through these hormone interactions, and through specific receptors identified on peripheral lymphocytes and other cells of the immune system, the catecholamines have been observed to have a variety of mostly suppressive effects on immune function (Berczi, 1986a). Wilkes et al. (1964) observed thymic involution after the

systemic administration of epinephrine. Lymphocyte depletion after stress in adrenalectomized rats was attributed to the increased production of norepinephrine in these animals (Rocha, 1985).

SUMMARY

Involution of the thymus gland is a normal occurrence with advancing age and is also observed precociously in certain strains of animals, or due to stress or other physiological imbalances. In some species thymic atrophy and subsequent hypertrophy is a seasonal occurrence. Thymic involution, and associated abnormalities in immune function, may be reversed by the thymotrophic factors. An understanding of these thymotrophic factors is the first step toward the eventual goal of being able to enhance thymus-related aspects of immune functions.

As discussed in this review the thymotrophic factors include the pituitary hormones GH and PRL, thyroid hormones, gonadectomy (reflecting decreased gonadal steroids), and adrenalectomy (reflecting decreased glucocorticoids). These factors have in common being part of, or affecting hormones which are a part of, the endocrine system. Further, those factors associated with thymic hypertrophy (GH, PRL, thyroxine) are all classified as peptide hormones, whereas those associated with thymic atrophy (gonadal steroids, glucocorticoids) are classified as steroid hormones. Receptors for all of these hormones have been demonstrated in thymic tissue. These observations demonstrate the importance of the interrelationship between the nervous, endocrine, and immune systems.

An evolution in the study of neuroendocrine-immune interactions is apparent. Early reports were concerned simply with the anatomical description of thymic hypertrophy. More recent studies are focusing on the function of the regenerated thymus and immune system since it cannot be assumed that better morphology is equivalent to better function.

The investigation of the actions of thymotrophic factors is complex because thymus structure and function are complex. There are many different cell populations and subpopulations to be discerned, and a given hormone may affect a certain subpopulation of cells and not another. With further elucidation of the mechanisms of action of the various thymotrophic agents more is learned about the immune system itself. Some of these thymotrophic agents are now being tested as immunoenhancers, and their clinical importance will almost certainly increase in the future.

SECTION II: GROWTH HORMONE TREATMENT STIMULATES THYMULIN PRODUCTION IN
AGED DOGS

GROWTH HORMONE TREATMENT STIMULATES
THYMULIN PRODUCTION IN AGED DOGS

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Clinical and Experimental Immunology 68:580-587, 1987

INTRODUCTION

Morphological involution and functional decline of the thymus normally occurs with advancing age (Delafuente, 1985; Schuurman et al., 1985). Histologically, the age-involuting thymus is characterized primarily by a decreased cortex:medullary ratio, reflecting low lymphocyte numbers and fatty infiltration. Cell-mediated immune mechanisms, which depend on the thymus as a source of differentiated effector cells, also decline with advancing age. The pattern of release of some of the adeno-hypophyseal hormones such as growth hormone (GH) is affected in aging, with an overall decrease in production with advancing age (Sonntag et al., 1983). The thymus gland has an endocrine component, the thymic epithelial cells, which produce several well-characterized peptides (Monroe and Roth, 1986). The general function of these peptides is to induce differentiation of pre-T lymphocytes and to modulate the function of more mature T lymphocytes (Incefy, 1983). One such peptide is thymulin, the zinc-conjugated form of facteur thymique serique (FTS). Plasma thymulin concentration has been shown to be significantly lower in aged versus young mice and humans (Fabris and Moccohegiani, 1985; Bach et al., 1975; Iwata et al., 1981).

The concurrent age-associated waning of hypophyseal and thymic hormone concentrations is significant in the light of reports that the nervous and immune systems are functionally and structurally linked. The thymus is probably not the primary site of the age-associated functional decline since, when grafted into a young recipient, an old thymus will regain at least part of its endocrine function, including increased

thymulin production (Bach and Beaurain, 1979; Fabris and Mocchegiani, 1985). A hypothalamo-hypophyseal-thymus circuit, including feedback control of thymulin production, has been proposed (Hall and Goldstein, 1983; Savino et al., 1983). Results of experiments testing the developmental and functional effects of thymectomy on the pituitary, or hypophysectomy on the thymus, support such a hypothesis (Fabris and Piantanelli, 1982; Pierpaoli and Sorkin, 1967).

We previously reported the results of treatment of immunodeficient dwarf puppies with bovine growth hormone (bGH) (Roth et al., 1984; Roth and Goff, 1985). These puppies, from a colony of inbred Weimaraners maintained at Iowa State University, had subnormal thymic development associated with a wasting syndrome that was usually fatal if untreated. Growth hormone release in response to clonidine administration was also low in these puppies. Clonidine provocation of GH release is used because GH is normally released in a pulsatile manner making random sampling meaningless. Therapy with bGH resulted in clinical improvement of the wasting syndrome and enhanced thymic development. Since diminished thymic morphology and function and a decreased provocative GH response are characteristic of normal aging, it was hypothesized that bGH treatment of old dogs would improve their thymic morphology and function. Preliminary studies in a diverse group of aged dogs indicated that bGH treatment tended to improve thymic morphology (Monroe et al., 1987). The purpose of this study was twofold: (1) to determine thymulin concentrations in plasma from dogs of various ages, (2) to evaluate the effect of bGH administration on: (a) thymic morphology, and (b) one measure of thymic function, plasma thymulin concentration. It was hypothesized that we

would observe a decline in plasma concentration of thymulin with advancing age and that bGH therapy would result in improved thymic morphology and increased thymulin production.

MATERIALS AND METHODS

Dogs

Thirty-three pure-bred female beagles of known age and medical history served as subjects for the present study. Dogs were purchased from Laboratory Research Enterprises, Inc., Kalamazoo, MI. All but the 'young' age group were retired breeders.

Thymulin concentrations in dogs of various ages

Subjects Dogs in this experiment were of three age groups: 'young' (five dogs, 4 months old), 'middle-aged' (10 dogs, 33 to 55 months old), and 'old' (18 dogs, 63 to 83 months old).

Assay Blood for the thymulin assay was collected into EDTA by jugular venipuncture between 0830 and 0930 hours. The blood collection tubes were immediately placed in an ice bath to preserve thymulin activity. The tubes were centrifuged at 4°C (750g); the plasma was removed and stored frozen at -70°C. Thymulin determinations were performed in the laboratory of Dr. G. S. Incefy according to the rosette inhibition assay of Dardenne & Bach (1975) with minor modifications as described by Iwata et al. (1981). The thymulin assay is based on the observation that rosette-forming cells in the spleens of thymectomized mice are less sensitive to azathioprine than are those from normal mice. After a 75 minute incubation at 37°C, plasma with thymulin activity restores to normal the sensitivity to azathioprine of rosette-forming cells from adult thymectomized mice, resulting in inhibition of rosette formation. This change has been used to establish a reproducible and quantitative bioassay for determination of circulating thymulin in plasma.

Data were converted to the \log_2 of the reciprocal of the titer for comparison.

Assay validation Immunoabsorption using a monoclonal antibody to FTS (MA-FTS) (Ohga et al., 1982) was performed in duplicate on six canine plasma samples containing high levels of thymulin activity as a validation step to ensure that thymulin was the active component in the bioassay. Briefly, plasma filtrates were incubated for 2 hours at 4°C with MA-FTS cross-linked to cyanogen bromide activated Sepharose 4B beads. After incubation, the suspensions were centrifuged and the activity of the supernatant evaluated by the rosette-inhibition assay. Using this procedure, thymulin is adsorbed by the anti-FTS immunosorbant, whereas other factors that may be active in the rosette-inhibition assay, particularly T lymphocyte derived allogeneic factor, remain unadsorbed in the supernatant. In addition, human growth hormone was analyzed at various concentrations in the bioassay because the exogenous hormone might be present in the plasma samples and could, theoretically, be active in the assay. Human GH was used in place of bGH, which was not available at the time of validation.

GH therapy

Subjects In order to evaluate the effects of GH therapy on thymic function, the middle-aged and old dogs were assigned to three treatment groups blocked by age (group A ['middle-aged']: 10 dogs, 33-55 months old; group B ['old-aged']: 10 dogs, 63-83 months old; group C ['old-aged']: eight dogs, 66-82 months old).

Protocol One-half of the dogs in each group were treated with pituitary derived bGH and the other half received bovine serum albumin

(BSA) as a control. Pituitary derived bGH was purchased from Dr. A. F. Parlow, Research and Education Institute, Torrance, CA. The BSA was purchased from Sigma Chemical Co., St. Louis, MO. Dogs in groups A ('middle-aged') and B ('old-aged') received 14 doses of either bGH or BSA at 0.1 mg/kg body weight given subcutaneously over 30 days as previously described (Roth et al., 1984). In preliminary studies, 'old' dogs showed no change in thymic morphology with this GH regimen, so dogs in group C ('old-aged') received daily subcutaneous injections of 0.1 mg/kg of either bGH or BSA for 38 days to determine the effects of more frequent GH administration. Necropsies were performed immediately after sodium pentobarbital euthanasia; tissue samples were collected and fixed as quickly as possible. Paraffin embedded sections (5um) were stained with hematoxylin and eosin.

Stereology In middle-aged dogs, where histomorphological assessment indicated differences between groups, two randomly selected regions on each of three slides from each dog were used for stereological evaluation of thymic morphology. The volume fraction of the various thymic compartments was determined by point-counting using a systematic lattice (Weibel, 1979; Elias et al., 1971). The cortex to medullary ratio was determined for each dog and compared between groups. Additionally, subjective histomorphological evaluation of the thymic tissue was performed and was replicated by a diplomate of the American College of Veterinary Pathologists. All morphological data were collected from coded slides so that the observer was not influenced by the knowledge of the treatment group or individual dog number.

RESULTS

Thymulin concentrations in aged dogs

Assay validation In each plasma sample in which immunoadsorption with MA-FIS was performed, the thymulin titer was decreased by at least 4-fold, indicating that the original activity was due to thymulin. Human growth hormone did not show activity in the assay.

Thymulin concentrations at various ages An age-associated decline in the plasma concentration of thymulin was detected (Figure 1). The thymulin concentration decreased from the young to the middle-aged groups ($P < 0.01$, Student's t-test), with no further discernible decrease with advancing age.

bGH treatment of aged dogs

Thymic morphology Four of the five middle-aged dogs treated with bGH had improved thymic morphology when compared to age matched BSA-treated dogs. Only one of the five BSA-treated, middle-aged dogs had thymic morphology similar to the majority of the bGH-treated dogs. As illustrated in Figure 2, the corticomedullary junctions of bGH treated dogs were more distinct and the lobule size was greater, reflecting an increase in the number of lymphocytes present. Stereological examination of thymus glands from the bGH-treated middle-aged dogs, as compared to the BSA-treated dogs, revealed: (a) an increase in the volume fraction of cortex but no change in medulla, and (b) an increase in the cortex to medullary ratio ($P = 0.07$, Student's t-test) (Figure 3). There was no detectable histomorphological difference between thymus glands from the

Figure 1. Thymulin titers in dogs from three different age groups.
Horizontal lines indicate the mean for each age group

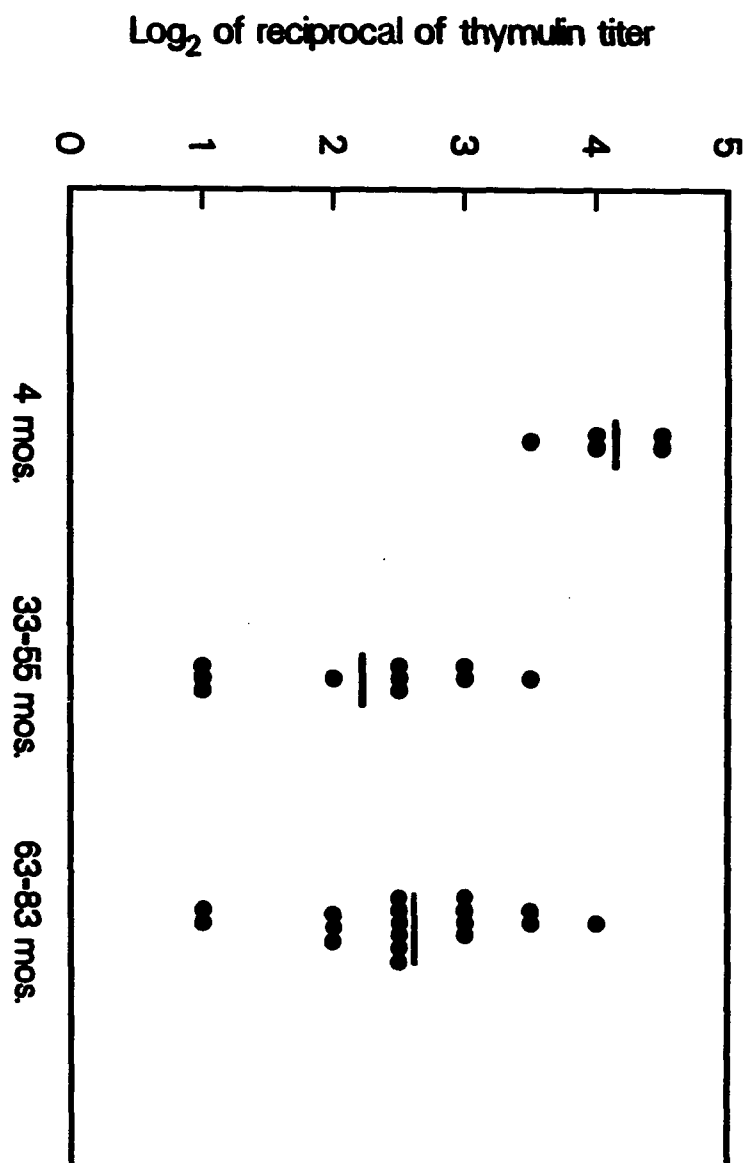
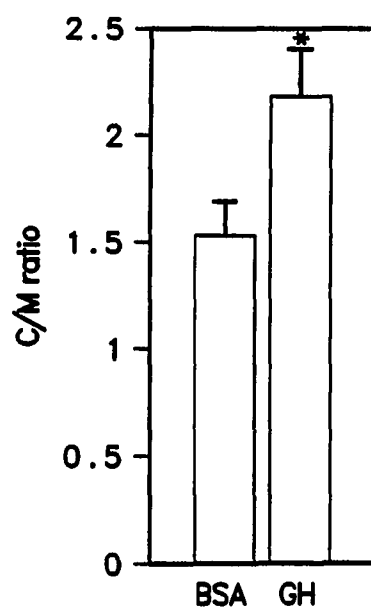
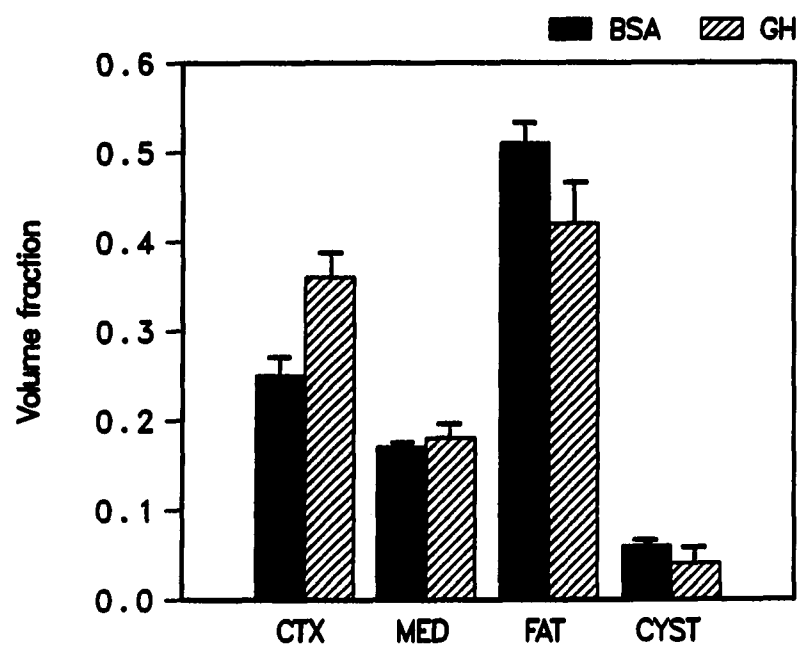


Figure 2. Light microscopic appearance of a representative thymus gland from middle-aged dogs treated with BSA (upper) or bGH (lower). Note the marked increase in cellularity and the distinct cortical and medullary areas in the thymus from the bGH treated dog. (bar = 500um)



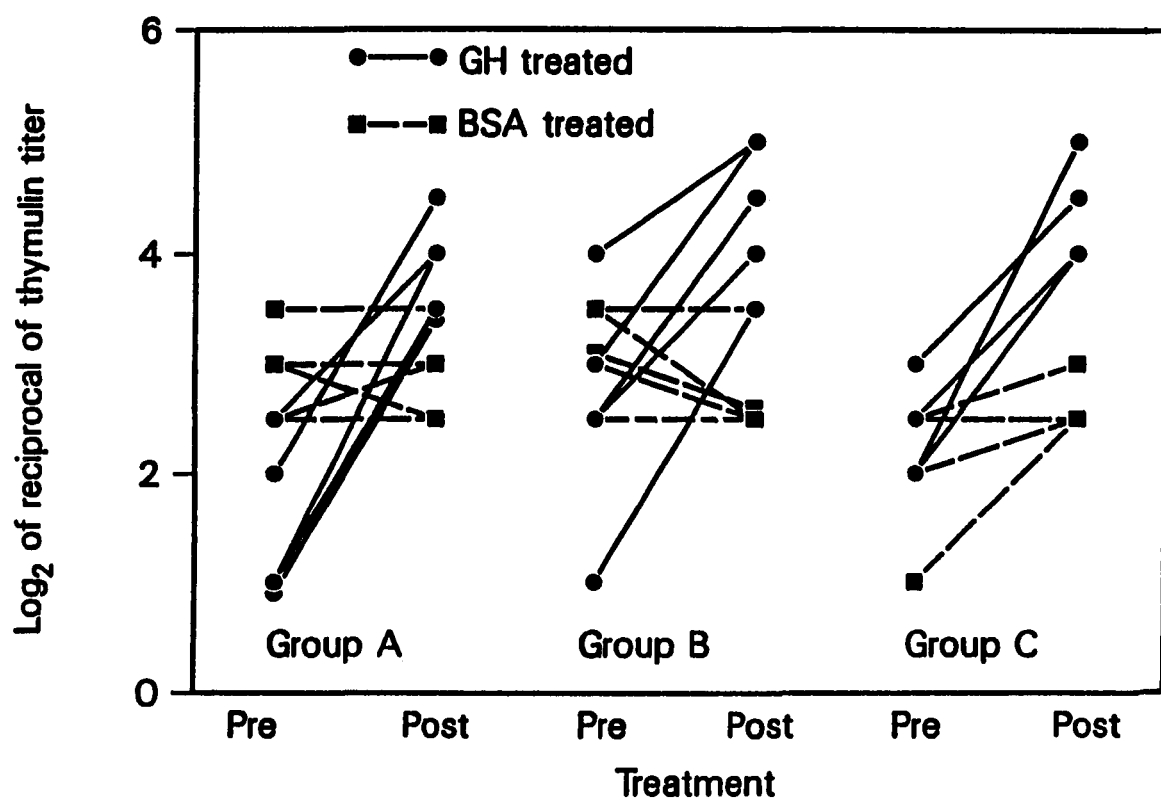
Figure 3. Mean (\pm SEM) volume fractions of various thymic constituents (upper) and cortex to medullary ratios (lower) in thymus glands from middle-aged dogs. CTX = cortex; MED = medulla; FAT = adipose tissue; C/M ratio = cortex to medullary ratio. CYST refers to regions of columnar to pseudostratified, ciliated columnar epithelium lined cysts, which are normally found in canine age-involuting thymus glands. * $P=0.07$, level of statistical significance of the difference between the bGH and BSA groups



bGH or BSA treated dogs in either of the old age groups (B and C) (data not shown).

Thymulin concentrations As demonstrated in Figure 4, bGH-treated dogs had significantly greater plasma thymulin concentrations than BSA-treated controls regardless of age ($P < 0.01$, Student's t-test). Most of the BSA treated dogs showed either no change or a decline in plasma thymulin concentration; only a few BSA treated dogs showed slight increases in thymulin titer.

Figure 4. Thymulin titers in middle-aged (group A) and old-aged (groups B and C) dogs treated with BSA or bGH. Groups A and B were injected 14 times in a 30 day period, as described in the text; group C received daily injections. The post-treatment plasma sample for each group was taken 30 days after the initiation of treatment. Plasma thymulin concentrations were increased in every dog treated with bGH



DISCUSSION

As expected, plasma thymulin concentrations decreased with age in dogs (Figure 1). Similar declines have been reported in rodents and humans, where an initial decrease is followed by relatively constant, but lower, thymulin concentrations with advancing age (Fabris and Mocchegiani, 1985; Bach et al., 1975; Iwata et al., 1981).

Improved thymic morphology was observed in four of the five bGH treated middle-aged dogs, confirming preliminary studies (Monroe et al., 1987). These changes were obvious at the gross and light microscopic levels (Figure 2). Stereological evaluation of the tissue sections reflected these changes (Figure 3), especially with regard to the cortex to medullary ratio ($P=0.07$). That these obvious changes, when evaluated by point-counting to determine volume fractions of various thymic components, were not highly statistically significant indicates the proportional growth of the different tissue components in the thymus. Such proportional morphological changes mask the actual changes in particular components of the organ when per-unit-volume analysis is used. To demonstrate the true change in the tissue components the measured volume fractions should be multiplied by the overall tissue volumes to indicate the absolute volumes (Loud and Anversa, 1984). However, the nature of the thymus, particularly the involuted thymus, precludes accurate measurement of the tissue volume, as other investigators have also realized (Khmel'nitskii et al., 1986). Measurements of thymic volume may be confounded by edema fluid (which may be present in the stress-involuted thymus) and adipose tissue; these may both be present grossly,

contributing to the total tissue volume, and then be lost to varying extents during tissue collection, handling, and routine processing before stereological measurements can be made. One of the bGH-treated dogs did not respond with greatly improved morphology, and one of the five BSA-treated dogs did have a thymus with better than expected morphology. There is a normal variation in the morphology of the thymus in individuals of the same age (Steirmann et al., 1985); factors that can cause changes in thymic morphology include stress, viral infections, and other hormones in addition to GH.

In contrast to the middle-aged dogs, there was no detectable histomorphological change in the thymus glands of the 'old' dogs, which could be interpreted as a loss of the ability to respond to bGH in advanced age. However, a change (or a lack of change) in thymic morphology does not prove increased or decreased thymic function; immunological or endocrine function must also be assessed. The present results indicate that bGH treatment did stimulate the endocrine function of the thymus, as measured by its thymulin production. As demonstrated in Figure 4, even the oldest dogs studied (which had no change in thymic morphology) had consistent increases in plasma thymulin concentrations. More frequent bGH injections (Group C) did not result in any further increase in thymulin titers (Figure 4).

Enhancement of thymus endocrine function after bGH treatment is significant, considering the immunostimulatory effects of thymulin. There are several conditions in animals and humans in which a decline in thymulin concentration has been documented. These include immune-related diseases (AIDS, DiGeorge's syndrome, severe combined immunodeficiency, and

chronic graft-versus-host disease), certain nutritional deficiencies (protein-calorie malnutrition, zinc or pyridoxine deficiency, and advanced anorexia nervosa), and some miscellaneous conditions (hypothyroidism, some asthmatic children and most Down's syndrome patients) (Grody et al., 1985; Atkinson et al., 1982; Chandra, 1980; Wade et al., 1985; Garaci et al., 1978; Franceschi et al., 1981; Incefy et al., 1986). Thymulin treatment in vivo, or in vitro treatment of lymphocytes, have yielded some positive results in conditions such as rheumatoid arthritis, systemic lupus erythematosus, and some types of immunodeficiencies in children (Faure et al., 1984; Bene et al., 1982). Thymulin treatment in aged individuals resulted in partial correction of age-associated immune deficiencies including lymphocyte-mediated cytotoxicity and interleukin 2 production (Bach, 1977; Schulof, 1985; Zatz and Goldstein, 1985). One problem with exogenous thymulin treatment is that the half-life of thymulin in the blood is short (less than 15 min.), perhaps due to proteolysis, binding to a carrier protein or some other clearance mechanism (Hadden and Keskiner Merriam, 1985; Savino et al., 1983). Treatment with GH may enhance immune function in aged individuals by causing a sustained elevation of plasma thymulin concentration and perhaps will stimulate increases in other thymic hormone concentrations as well.

It has been hypothesized that the decline of thymulin concentration with age is largely dependent on age-associated endocrinological imbalances. It may therefore be more effective to treat with a hormone derived from a higher level in the neuroendocrine hierarchy (pituitary derived) that would be likely to have a variety of actions on thymic function as opposed to simple replacement of the peripherally deficient

hormone, thymulin. Other pituitary hormones, and hormones under pituitary control, also affect thymic morphology and thymic endocrine function. Prolactin and growth hormone-secreting GH₃ pituitary adenoma cells implanted into aged rats improve thymic structure and T cell function (Kelley et al., 1986). The thyroid hormone, thyroxine, which is under the direct influence of the pituitary hormone thyroid stimulating hormone (TSH), has been shown to increase thymulin production in aged animals although improvement in thymic morphology was not reported, and an increase in thymulin was not observed in very old animals (Fabris and Mocchegiani, 1985; Fabris et al., 1982b; Fabris et al., 1982a). Human hyper- and hypothyroid patients have thymulin concentrations that are higher and lower, respectively, than in normal patients; a significant correlation was found between thymulin and T3 and T4 concentrations (Fabris et al., 1986). However, thyroxine can affect both prolactin and growth hormone production (Peake et al., 1973; Martial et al., 1977). Rather than acting singly, it seems likely that these and other hormones interact in an as yet undefined manner to produce their effects on the immune system.

We have demonstrated that GH treatment not only improves thymic morphology in middle-aged dogs, but also thymic function as evidenced by increases in thymulin levels even in the oldest dogs studied. The results suggest that exogenous GH may be useful for restoration of some immune functions in aged individuals.

SECTION III: THE EFFECT OF ORAL CLONIDINE ON GROWTH HORMONE RELEASE,
THYMIC STRUCTURE AND IMMUNE FUNCTION IN AGING DOGS

THE EFFECT OF ORAL CLONIDINE ON GROWTH HORMONE RELEASE,
THYMIC STRUCTURE AND IMMUNE FUNCTION IN AGING DOGS

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INTRODUCTION

The structure and function of the thymus change with age, beginning at about the time of puberty (Delafuente, 1985; Schuurman et al., 1985). Thymic involution in the dog is characterized by a decrease in the cortex to medullary ratio (reflecting a decrease in the lymphocyte population), and increases in adipose tissue and the incidence of thymic cysts. Cell-mediated immune function, which depends on the thymus as a source of differentiated effector cells, also declines with age (Weksler et al., 1978; DeKruyff et al., 1980; Cowan et al., 1981; Schultz, 1984). Thymic endocrine function, as assessed by the secretion of various thymic hormones, is decreased in aged versus young animals, including humans (Coesterom and Kater, 1982; Hirokawa et al., 1982; Fabris and Mocchegiani, 1985). Since the secretion of growth hormone (GH) from the adenohypophysis also diminishes with age (Sonntag et al., 1983) investigations of the relationship between GH, thymus function, and aging have been undertaken. A hypothalamo-hypophyseal-thymus circuit, including feedback control of thymulin (a thymic hormone) production, has been proposed (Hall and Goldstein, 1983; Savino et al., 1983). This hypothesis is supported by the results of experiments testing the developmental and functional effects of thymectomy on the pituitary or of hypophysectomy on the thymus (Pierpaoli and Sorkin, 1967; Fabris and Piantanelli, 1982).

We have previously reported the thymotropic and immunoenhancing effects of bovine GH (bGH) treatment in immunodeficient dwarf Weimaraner puppies and in aging female beagle dogs (Roth et al., 1984; Roth and Goff, 1985; Goff et al., 1987). Administration of bGH was thymotropic in

middle aged (33 - 55 months) but not in old aged (63 - 83 months) dogs (Goff et al., 1987). However, thymus endocrine function, as measured by thymulin secretion, was enhanced in every bGH treated dog, regardless of age or of the bGH effect on thymic morphology (Goff et al., 1987).

The long term administration of exogenous GH has two drawbacks: bGH must be administered by injection, and the subject is likely to develop antibodies to the foreign protein, which would block its effect. It is more desirable to cause the release of endogenous GH which would more closely approximate the true physiological state of a younger subject. In an effort to induce endogenous GH secretion in aging dogs, Morrison (1987) assessed various secretagogues of GH, including arginine, ornithine, and clonidine hydrochloride. Clonidine is an alpha 2 adrenergic agonist and is used in the provocative testing of GH secretion in humans (Hunt et al., 1986). Of the secretagogues tested, clonidine was the most reliable stimulant of GH secretion, and an optimal dosage for oral administration was determined (Morrison, 1987). However, the dosage regimen (100 ug/kg, twice daily in the food) resulted in an apparent desensitization, or down regulation, of the pituitary GH response to clonidine administration; GH secretion and immune function were comparable in experimental and control groups by day 30 of the study. The purpose of this study was to evaluate the effect of intermittent clonidine administration on GH release, thymic morphology and various parameters of immune function in aging dogs. It was hypothesized that intermittent administration of clonidine to aging dogs would avoid desensitization, and result in a consistent release of GH and an enhancement of immune function lasting throughout the study period.

MATERIALS AND METHODS

Subjects and experimental design

Eighteen female beagles, 47 - 67 months old, served as subjects for this study. All dogs were retired breeders, of known medical history, purchased from Laboratory Research Enterprises (Kalamazoo, MI). The dogs were fed Hill's Science Diet maintenance formula (Hill's Pet Products, Inc., Topeka, KS) at 700 calories per day, which is an appropriate maintenance ration for caged dogs of this age and weight range (Lewis et al., 1987).

The dogs were randomly assigned to one of three groups blocked by age and weight. Dogs in group 1 served as controls by receiving empty gelatin capsules. Group 2 dogs were given clonidine every second day (total of 15 doses). Dogs in group 3 received clonidine every third day (total of 10 doses). Clonidine HCl tablets (American Therapeutics, Inc., Bohemia, NY) were placed within gelatin capsules and were administered orally at approximately 0900 hours at a dosage of 100 ug/kg.

The dogs were euthanatized on day 30 of the study period by sodium pentobarbital injection; thymic tissue was collected as quickly as possible and fixed in 10% neutral buffered formalin.

Assessment of growth hormone release

Blood collection Blood to be assayed for growth hormone content was collected at the beginning (day 1), middle (day 15 or 16), and end (day 28 or 29) of the study period. Sample collection began between 0830 and 0900 hours, just prior to (time 0), and 30, 60 and 90 minutes after administration of the gelatin capsules. Venous blood samples were

collected into EDTA, put immediately into crushed ice, centrifuged at 4°C, and stored at -70°C until assayed.

Radioimmunoassay for canine growth hormone Plasma GH content was determined by a double antibody radioimmunoassay specific for canine growth hormone. Pituitary derived, purified canine GH (cGH) for iodination and standards, monkey anti-cGH gamma globulin, and procedural details for the assay were acquired from Dr. A. F. Parlow (Pituitary Hormones and Antisera Center, Harbor-UCLA Medical Center, Torrance, CA). Briefly, cGH was solubilized and then immediately iodinated to low specific activity using IODO-BEADS (Pierce Chemical Co., Rockford, IL). Iodinated cGH was collected after being separated over a Sephadex G-100 column.

For the radioimmunoassay, 0.05 M PBS, cold standard or unknown plasma sample, iodinated cGH and monkey anti-cGH gamma globulin (1:31,250) were mixed together and incubated in tubes for 18 - 24 hours at room temperature. Goat anti-monkey gamma globulin (1:12), normal monkey serum (1:40) (both from Antibodies Inc., Davis, CA) and polyethylene glycol were added to each sample tube. After an incubation of two hours at room temperature, the tubes were centrifuged at 1500 x g at 4°C. The supernatant was removed and the radioactivity in the pellet was determined using a gamma counter.

Assessment of thymic morphology

Fixed thymic tissue collected at necropsy was processed and at least three tissue samples per subject were embedded in paraplast. Sections, 4 - 6 um thick, were stained with hematoxylin and eosin for examination at the light microscopic level. All morphological data were collected from

slides that were coded so that the identity of the subject and group were unknown to the observer. Subjective analysis of the degree of age involution was based on several parameters known to change progressively with increasing age, as shown in Table 1. An age-involuted thymus was defined as having an increase in the amount of fatty infiltration, discontinuity of thymic lobules, presence and extent of thymic cysts and a decreased cortex to medullary ratio. The thymus of each subject was then assigned a score from one to five; a score of 1 was assigned to a thymus with essentially no signs of age involution, whereas a score of 5 represented a thymus with advanced age involution. The subjective histomorphological evaluation of the thymic tissue was performed and then was replicated by a diplomate of the American College of Veterinary Pathologists (L.H.A.).

Hematology and clinical chemistry

Hematologic and clinical chemistry procedures were performed by the Clinical Pathology Section of the College of Veterinary Medicine, Iowa State University (Ames, IA) on serum samples collected from each dog at the beginning, middle, and end of the experimental period. The parameters measured included total and differential white blood cell count, packed cell volume, total serum protein, blood urea nitrogen (BUN), serum alkaline phosphatase, serum glutamic-pyruvic transaminase, serum albumin and blood glucose.

Assessment of primary antibody response

Blood collection One week after clonidine administration had begun each dog was immunized with tetanus toxoid and heat-killed strain 19 of Brucella abortus (B. abortus). Serum was collected prior to

Table 1. Explanation of thymic morphology scoring system

Score	C/M ^a junction	C/M ratio	IL ^b septae width	Thymic corpuscles	adipose tissue	cysts
1	distinct	1:1 or >	+ ^c	++++	+	-/+ ^d
2	distinct	1:1	++	+++	++	-/+
3	slightly indistinct	<1:1	+++	+++	++ to +++	-/+
4	indistinct	<<1:1	++++; lobules not contiguous	+ to ++	+++	++; with surrounding lymphocytes
5	indistinct	<<1:1	++++; lobules not contiguous	- to +	++++	++++; many without surrounding lymphocytes

^aCortex to medullary (C/M).

^bInterlobular (IL).

^cBased on a five '+' system, one + being the least, five +'s being greatest.

^dThymic cysts are rare in young dogs (-) but may be present in the regenerated thymus gland in aging dogs (+).

immunization and weekly thereafter for the duration of the experiment.

Brucella abortus The serum antibody titer to B. abortus was determined using the standard tube agglutination procedure. Standard Tube Test Antigen was obtained from the National Veterinary Services Laboratory (Ames, IA). The titer was expressed as the \log_2 of the inverse of the titer.

Tetanus toxoid The tetanus toxoid titer was determined using an enzyme-linked immunosorbent assay (ELISA) procedure. Tetanus toxoid was bound to a 96-well microtiter plate (Immulon 2, Dynatech Labs, Alexandria, VA) and serial 2-fold dilutions of serum, beginning with a 1:10 dilution, were made in the wells. A peroxidase-conjugated goat anti-canine IgG (heavy and light chain specific) was used to detect the presence of bound antibody.

Immunoglobulin quantitation

Serum samples to be assayed for immunoglobulin content were collected from each dog at the beginning and at the end of the experimental period. The total concentration of serum IgG, IgM, and IgA was measured using radial immunodiffusion kits (ICN ImmunoBiologicals, Lisle, IL) specific for the canine immunoglobulins.

Lymphocyte response to mitogens

Venous blood was collected from each dog into acid citrate dextrose (ACD) once per week (beginning one week prior to the start of clonidine administration). The blood samples were collected at approximately 0900 hours, prior to feeding. Lymphocyte blastogenesis in response to phytohemagglutinin (PHA), concanavalin A (Con A) and pokeweed mitogen (PWM) was performed on Histopaque 1077 (Sigma Chemical Company, St. Louis,

MO) purified lymphocytes as previously described (Roth et al., 1984).

Briefly, mitogen-stimulated lymphocytes were incubated for 48 hours before the addition of [^3H]-thymidine. After 16-18 hours additional incubation, the cultures were harvested and prepared for liquid scintillation counting.

RESULTS

Growth hormone release

Four of the six dogs in each treatment group responded to clonidine with GH release, although this response was inconsistent. On day 1, only three of the 12 dogs receiving clonidine responded by secreting GH (Figure 1a). Peak release of GH occurred 60 or 90 minutes after clonidine administration. On day 15, five of the 12 dogs receiving clonidine responded by releasing GH (Figure 1b); by day 30, clonidine administration resulted in GH release from seven dogs (Figure 1c). Those dogs responding to clonidine on day 1 continued to respond by GH release throughout the study period. There was no statistically significant difference between groups in the amount of GH released except 60 minutes after clonidine administration on day 30, for group 2. Control dogs, which had received empty gelatin capsules, did not respond with GH secretion.

Thymic morphology

The morphology scores assigned to each thymus sample are given in Table 2 along with references to photographs showing the light microscopic appearance of the glands (Figures 2 - 4).

The mean score for thymic morphology in group 2 dogs was less than that for dogs in groups 1 and 3, which were comparable. Five of the six dogs in group 2 had thymus glands with morphology better than expected for dogs of this age group. Three of the dogs in group 1 and two of the dogs in group 3 had thymic morphology better than expected for their age group.

Figure 1. Mean (\pm SEM) plasma growth hormone concentration before (0 minutes) and after (30, 60, and 90 minutes) oral clonidine. Control dogs received empty gelatin capsules; dogs in groups 2 and 3 received clonidine tablets in gelatin capsules every second or third day, respectively. There was no significant difference between groups in the plasma GH concentration on day 1 (A) or day 15 (B); at day 30 (C) dogs in group 2 released significantly more GH 60 minutes after clonidine administration as compared to control dogs (* $P < 0.05$, by analysis of variance)

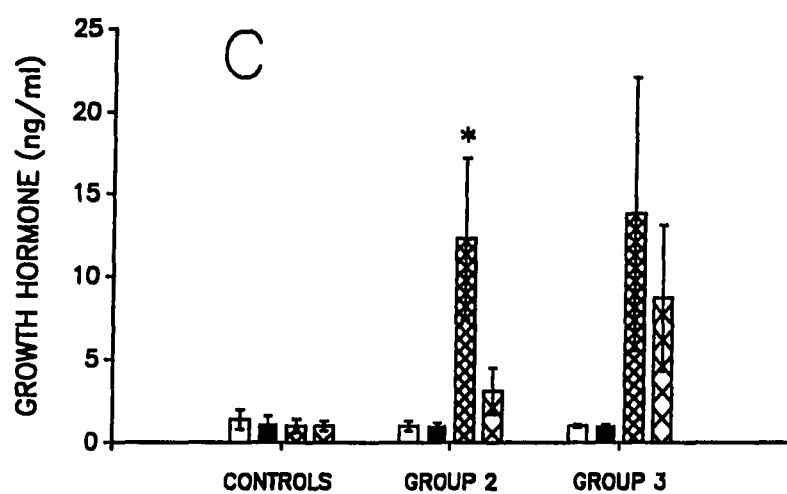
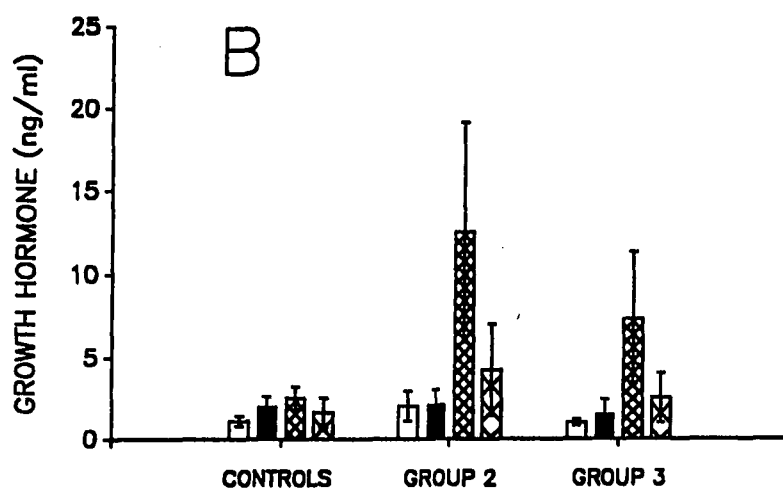
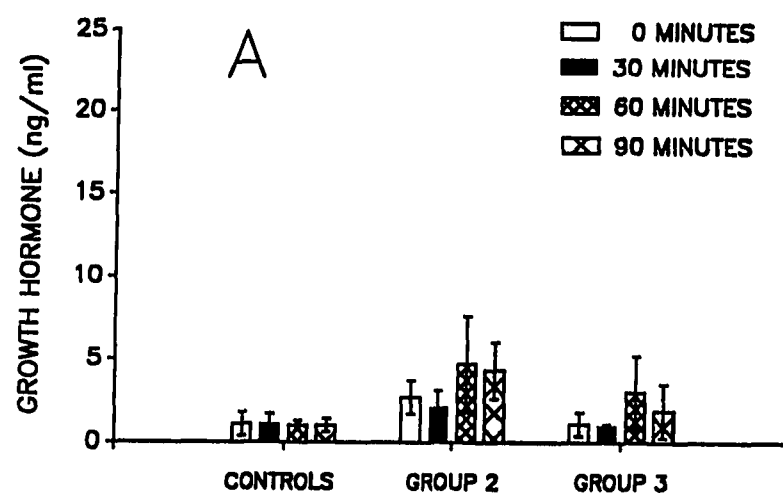


Table 2. Thymic morphology scores by group, with Figure references

Group	Animal	Score 1 ^a	Score 2	Mean Score	Figure
1	1223	5	4	4.5	2a
	1226	3.5	4	3.75	2b
	1231	2	2	2	2c
	1232	3.5	3	3.25	2d
	1235	4.5	4	4.25	2e
	1237	4	4	4	2f
				mean = 3.63	
2	1222	2	2	2	3a
	1224	3.5	3	3.25	3b
	1227	2.5	3	2.75	3c
	1228	2.5	3	2.75	3d
	1230	4	4	4	3e
	1234	2	2.5	2.25	3f
				mean = 2.83	
3	1220	2.5	2	2.25	4a
	1221	5	4.5	4.75	4b
	1225	5	4	4.5	4c
	1229	4	4	4	4d
	1233	2	2.5	2.25	4e
	1236	4.5	4	4.25	4f
				mean = 3.67	

^aScoring was assigned according to the guidelines presented in Table 1.

Figure 2. Light microscopic appearance of thymus glands from group 1 dogs (controls). Thymic morphology scores are listed in Table 2. (bar = 500um)



2b



2c



2d



2e



Figure 3. Light microscopic appearance of thymus glands from group 2 dogs (clonidine administered every second day). Thymic morphology scores are listed in Table 2. (bar = 500um)

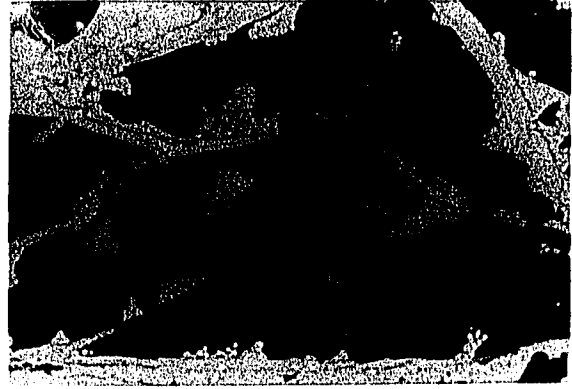
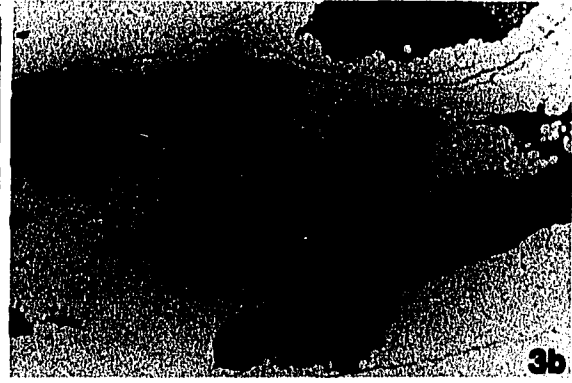
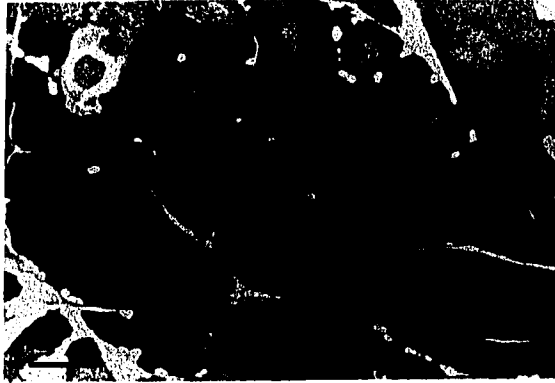


Figure 4. Light microscopic appearance of thymus glands from group 3 dogs (clonidine administered every third day). Thymic morphology scores are listed in Table 2. (bar = 500um)



Hematology and clinical chemistry

Values for the various clinical chemistry parameters, complete blood counts, and differentials did not vary from normal during the study period (individual data not shown) except in one animal. A group 2 dog had concentrations of serum alkaline phosphatase and serum glutamic pyruvic transaminase (SGPT) which were greatly elevated at day 15. By day 30 her alkaline phosphatase concentration had returned to normal; the SGPT concentration was greatly decreased but still was slightly elevated above normal.

While all clinical chemistry results were within normal ranges significant differences between groups were detected. As compared to controls, significantly lower mean concentrations of blood urea nitrogen (BUN) were detected in group 2 dogs at days 15 and 30, and in group 3 dogs at day 15 (Figure 5a). Mean blood glucose concentrations in group 2 and 3 dogs were significantly lower than in control dogs at day 30 (Figure 5b). There were no significant differences detected between groups in complete blood counts or differentials.

Primary antibody response

Brucella abortus The mean \log_2 titer of anti-B. abortus antibody for each group at each date tested is given in Table 3. Dogs in groups 2 and 3 developed higher mean \log_2 titers than the control dogs. This difference was significant at the $P < 0.10$ level for day 22 (two weeks post-immunization).

Figure 5. Mean (\pm SEM) plasma concentration of blood urea nitrogen (upper) and blood glucose (lower). * $P < 0.05$, ** $P < 0.01$ as compared to controls, by analysis of variance

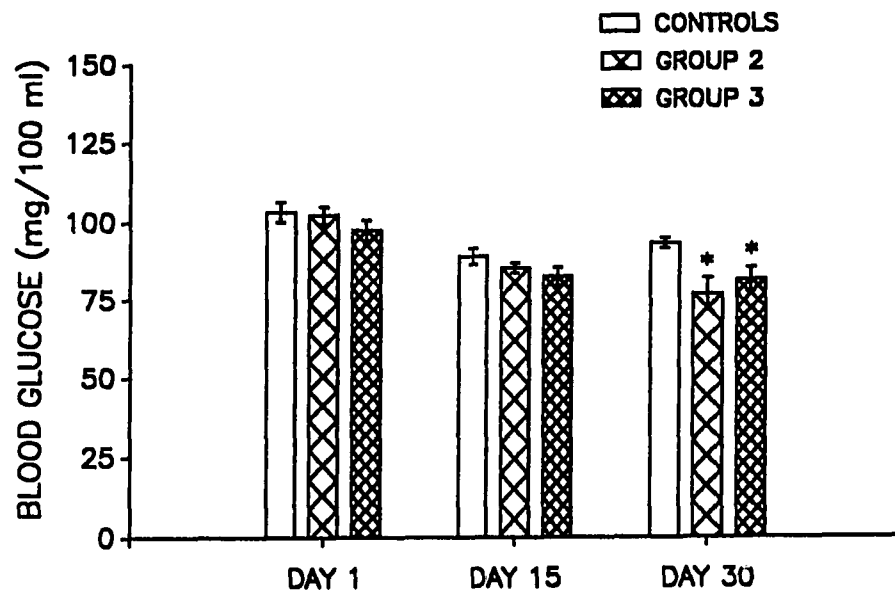
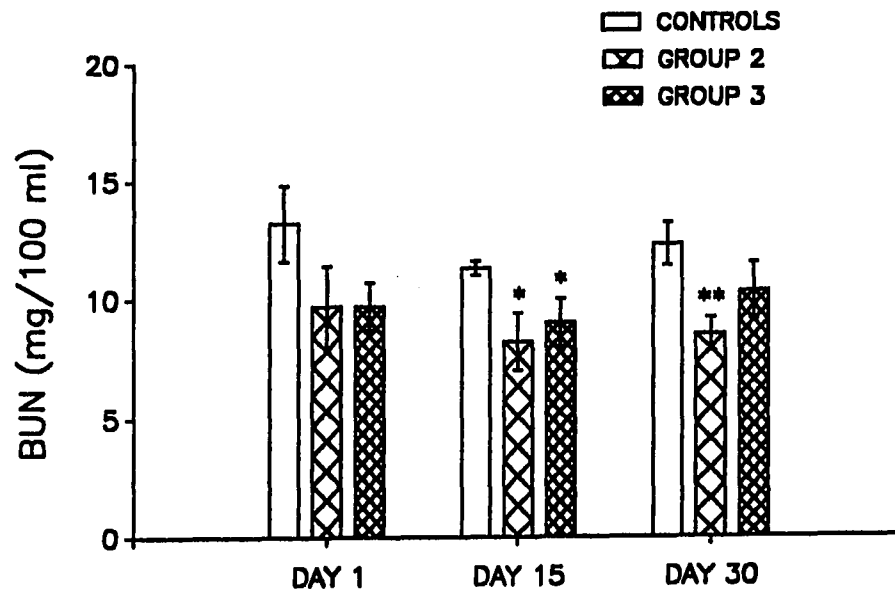


Table 3. Mean \log_2 of the inverse of the titer of anti-B. abortus antibody by group

Day	Group 1	Group 2	Group 3
8 ^a	0 \pm 0	0 \pm 0	0 \pm 0
15	8.82 \pm 0.43 ^b	9.49 \pm 0.17	8.99 \pm 0.56
22	8.66 \pm 0.42	9.82 \pm 0.34*	9.82 \pm 0.34*
29	8.49 \pm 0.48	8.99 \pm 0.21	9.32 \pm 0.45

^aDogs were immunized to heat-killed strain 19 of B. abortus on day 8.

^bMean \pm standard error of the mean.

* $P < 0.10$ when compared to group 1, by analysis of variance.

Tetanus toxoid The mean \log_2 titer of anti-tetanus toxoid antibody for each group at each date tested is given in Table 4. There were no significant differences between groups in their primary antibody responses to tetanus toxoid.

Immunoglobulin quantitation

The mean serum concentrations of IgG, IgM, and IgA, as measured by radial immunodiffusion, are presented in Table 5. There were no significant differences detected between groups in the serum concentration of the immunoglobulin classes tested.

Lymphocyte response to mitogens

No significant differences were detected between groups in the lymphocyte response to mitogens (Table 6). Results were found to be highly variable between dogs; individual dogs were consistently either high or low responders by this assay (data not shown).

Table 4. Mean \log_2 of the inverse of the titer of anti-tetanus toxoid antibody by group

Day	Group 1	Group 2	Group 3
8 ^a	0 \pm 0	0 \pm 0	0 \pm 0
15	0 \pm 0	0 \pm 0	0 \pm 0
22	8.49 \pm 1.01 ^b	8.32 \pm 0.52	8.32 \pm 1.03
29	9.16 \pm 0.79	8.66 \pm 0.76	9.16 \pm 0.65

^aDogs were immunized to tetanus toxoid on day 8.

^bMean \pm standard error of the mean.

Table 5. Mean serum concentration of IgG, IgM, and IgA on days 1 and 29, as measured by radial immunodiffusion

Group	Day	IgG	IgM	IgA
1	1	2458.3 \pm 232.5 ^a	95.0 \pm 7.3	18.2 \pm 2.9
	2	3233.3 \pm 518.8	96.2 \pm 9.9	17.0 \pm 2.9
2	1	2100.0 \pm 227.7	130.2 \pm 17.1	26.7 \pm 8.8
	2	2766.7 \pm 391.3	120.2 \pm 18.5	19.3 \pm 4.1
3	1	2616.7 \pm 332.1	125.2 \pm 16.1	26.0 \pm 7.8
	2	2500.0 \pm 335.7	135.7 \pm 28.3	20.2 \pm 3.8

^aConcentration of immunoglobulin in ng/ml \pm standard error of the mean.

Table 6. Mean lymphocyte response to mitogens, by group, prior to and during the clonidine treatment period

Mitogen	Period	Group 1		Group 2		Group 3	
none	1 ^a	370.6 ±	51.4	467.8 ±	117.0	463.2 ±	61.9
	2 ^b	278.4 ±	48.2	383.5 ±	80.6	284.9 ±	43.5
PHA SI	1	31.8 ±	8.9	36.4 ±	13.4	22.2 ±	3.5
	2	21.9 ±	5.1	30.4 ±	7.8	22.7 ±	3.8
PHA dcpm	1	13090.0 ±	5460.2	20220.6 ±	8448.1	9707.1 ±	2171.2
	2	7209.0 ±	2299.1	12556.7 ±	3613.8	6972.3 ±	1842.1
Con A SI	1	43.2 ±	10.6	41.1 ±	12.3	30.3 ±	4.9
	2	39.4 ±	6.5	49.9 ±	11.3	43.9 ±	5.7
Con A dcpm	1	17793.5 ±	6708.6	21928.7 ±	8143.2	14075.8 ±	3554.3
	2	12131.6 ±	3309.8	19404.9 ±	5032.8	13415.2 ±	2919.9
PWM SI	1	26.8 ±	5.2	22.9 ±	6.7	18.1 ±	3.3
	2	30.5 ±	5.5	34.6 ±	7.8	31.4 ±	4.9
PWM dcpm	1	10242.5 ±	2903.6	12729.5 ±	5166.6	8551.4 ±	2288.3
	2	9720.0 ±	2774.5	14084.3 ±	2774.5	10366.6 ±	2613.4

^aEach period 1 value represents the mean ± the standard error of the mean of two weekly determinations on six animals per group, prior to the clonidine treatment period.

^bEach period 2 value represents the mean ± the standard error of the mean of four weekly determinations on six animals per group, during the clonidine treatment period.

DISCUSSION

The inconsistent and delayed GH response to clonidine (Figure 1a - c) was an unexpected finding. In a previous study (Morrison, 1987), an equivalent dosage of clonidine resulted in a GH peak 30 minutes after administration in each dog. Morrison (1987) administered liquid clonidine by placing it on dry dog food. In the present study, enteric coated clonidine tablets were placed in gelatin capsules and administered orally, prior to feeding. As Figure 1 shows, the GH response to clonidine was delayed until 60 minutes after administration. While gelatin capsules dissolve quickly in the stomach, enteric coated tablets pass through the stomach and do not dissolve until they reach the small intestine (Gilman et al., 1985). The observed delay in the GH response to clonidine may be attributed to the delay in digestion and absorption of the clonidine in tablet form. The inconsistent response to clonidine within treatment groups might reflect variability in the digestive function between individual dogs. A greatly delayed GH response to clonidine would not be detected if it occurred later than 90 minutes post-administration when the last blood sample was collected.

Those dogs that responded to clonidine on day 1 continued to respond throughout the study period. In a previous study (Morrison, 1987) dogs became tolerant by day 30 to a similar dosage of clonidine administered twice daily. Tolerance, or down regulation, in response to chronic administration of certain drugs is a clinically recognized phenomenon that may involve both metabolic and receptor changes (Snyder, 1979). The results of the present study indicate that intermittent administration of

clonidine (once every two to three days) may overcome the problem of down regulation of the GH response.

Thymic morphology was better than expected for dogs of this age in five of the six dogs in group 2, and in two of the six dogs in group 1, indicating the great variability in thymus morphology between individual dogs. By means of subjective analysis of thymic morphology according to the parameters detailed in Table 1, group 2 dogs had a mean thymus score that was less than those for either the control group or group 3 (Table 2), although this was not statistically significant ($P>0.05$). A lower score on our scale of 1 to 5 (Table 1) corresponds to thymic morphology that would be observed in younger dogs before changes associated with age involution are apparent.

Dogs receiving clonidine had decreased serum BUN and glucose concentrations, as compared to control dogs, by the middle of the study period (Figure 5). Factors that may be involved in decreased BUN concentrations include increases in glomerular filtration rate (GFR) and protein anabolism. Clonidine is an alpha adrenergic agonist that reduces renal vascular resistance without affecting renal blood flow or GFR (Gilman et al., 1985). The anabolic actions of GH include a reported decline in BUN (Hutchings, 1959; Feldman and Nelson, 1987). Thus, it appears that GH was the most likely mediator of BUN changes in this study.

Blood glucose concentration is lowered in response to increased insulin release from the pancreas. Growth hormone promotes hyperglycemia primarily through insulin antagonism, but prolonged elevation of plasma GH reportedly results in a secondary hyperinsulinemia and subsequent diabetes mellitus (Feldman and Nelson, 1987). Some of the effects of GH are

mediated by the somatomedins (especially somatomedin C) which are similar to proinsulin and thus are also known as proinsulin-like growth factors (Feldman and Nelson, 1987). Due to the variety of possible GH effects on carbohydrate metabolism, further studies of this kind should include glucose tolerance tests, and measurement of blood somatomedin and insulin concentrations.

The function of the immune system is multifaceted and cannot be inferred from thymic morphology alone. For this reason we performed tests that would measure the function of both the cellular and humoral arms of the immune system. The results of these assays of immune function in the present study were varied. The primary antibody response to B. abortus, but not to tetanus toxoid, was enhanced in clonidine treated dogs (Tables 3 - 4). While antibody production is a B-cell (plasma cell) function, T-lymphocytes assist by providing help (T helper cells) to the B-lymphocytes. Thus, any agent affecting the function of the thymus or T-lymphocytes may ultimately affect antibody production. The primary antibody response (IgM) to sheep red blood cells, as measured by a plaque assay, is depressed by α_2 adrenergic agonists such as clonidine (Sanders and Munson, 1985) but is enhanced by GH (Deschaux et al., 1980). The Brucella tube agglutination test detects primarily IgM (which has greater agglutinating ability than IgG) whereas the ELISA for anti-tetanus toxoid antibody detects both IgG and IgM in this study (by means of secondary antibody to canine IgG, heavy and light chain specific). The fact that the primary antibody response to B. abortus, but not to tetanus toxoid, was enhanced in this study may indicate variable affects of clonidine and/or GH on antibody production.

Other parameters of immune function tested were not affected by clonidine treatment (Tables 5 - 6). In contrast, Morrison (1987) found enhanced blastogenic response to mitogens in a similar study. The inconsistent GH response, and the high variability within groups in blastogenic response, likely contributed to the negative finding in this study.

In ongoing investigations, we are combining an intermittent dosage regimen (every second day) with oral administration of liquid clonidine in hopes of optimizing delivery of clonidine. Results of the study reported here and previous research indicate that clonidine administration is associated with enhancement of some immune functions in aging dogs.

**SECTION IV: THYMIC CYSTS IN AGING DOGS: LIGHT AND ELECTRON MICROSCOPIC
ANATOMY, HISTOCHEMISTRY, AND IMMUNOCYTOCHEMISTRY**

THYMIC CYSTS IN AGING DOGS: LIGHT AND ELECTRON MICROSCOPIC
ANATOMY, HISTOCHEMISTRY, AND IMMUNOCYTOCHEMISTRY

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INTRODUCTION

The presence of cysts in the canine thymus was first reported over 100 years ago (Watney, 1882). Cyst-like structures in the thymus have been reported in several mammalian, amphibian and avian species (Oksanen, 1968; Dustin, 1911; Hammar, 1921). In dogs, the cyst epithelium is most often observed to be pseudostratified with cilia, although epithelial height may vary even within a single cyst (Oksanen, 1968). At the electron microscopic level, cyst epithelial cells in rats have been categorized into mucous secreting cells, electron-dense cells containing granules, and basal cells; a secretory function was hypothesized based on the ultrastructure of the cyst epithelium (Meihuizen and Burek, 1978). Dardenne et al. (1983) observed many secretory granules, some with electron dense cores, in the thymic cysts of the db/db mouse mutant. It was hypothesized that these granules could represent abnormal storage of a secretory product (Dardenne et al., 1983; Nabarra and Andrianarison, 1987). The ultrastructural appearance of thymic cyst epithelium in the dog has not been previously reported.

Some authors, noting the active and secretory nature of the cyst epithelium, have proposed that these cells have a special function, probably of an endocrine significance (Arnesen, 1958; Oksanen, 1968) or possibly involved with immune responsiveness (Kirkman and Kirkman-Liff, 1985). With histochemical techniques, Oksanen (1968) and Newman (1971) found the luminal contents of thymic cysts to contain neutral and acidic mucins, and complex sulphated 'mucosubstances'; the cyst epithelia stained positively for six different enzymes (Oksanen, 1968).

Hypothesized functions for thymic cysts have been proposed not only on the basis of cyst morphology, but also after observing naturally occurring mutants or the results of experimental manipulation. Factors such as age, stress, various hormones and diseases, which are known to affect other parameters of thymic anatomy, also affect the incidence of thymic cysts.

There is a positive correlation between aging and cyst incidence in the dog (White, 1942; Bargmann, 1943; Newman, 1971). Oksanen (1968) found the incidence of thymic cysts to be only partly dependent on age in dogs; cyst incidence was most correlated with the 'accidental' involution observed in stress such as that associated with disease or hunger. Thus, disease and other stressors, with their associated effects on endocrine function, seem to cause the precocious appearance of thymic cysts.

The effects of various hormones on thymus morphology and immune function have recently been reviewed (Berczi, 1986b; Goff, 1988). The relationship between cyst incidence and the hormonal state of the subject seems to be multi-faceted. In frogs, birds, and ground squirrels, thymic structure and cyst incidence has been observed to vary seasonally (Plytycz and Bigaj, 1983; Kendall, 1981; Shivatcheva and Hadjioloff, 1987; Glick, 1984). The hormones involved with the seasonal changes in thymic cysts have not been elucidated. In wild birds, peak thymic development was observed to follow the annual breeding season and was hypothesized to be related to increased thyroid activity associated with molting (Hohn, 1956; Glick, 1984).

Investigations of naturally occurring hormonal defects and exogenous hormonal treatments also demonstrate the endocrine effect on cyst

incidence. The thymus of db/db (diabetic) mouse mutants, which have congenitally low levels of prolactin, growth hormone, and thymulin (one of the thymic hormones), undergoes a precocious involution and contains cysts, which are rare in normal mice (Dardenne et al., 1983). Treatment with exogenous estrogen in hamsters (Kirkman and Kirkman-Liff, 1985) and rats (Glucksmann and Cherry, 1968) caused the precocious appearance of thymic cysts. With a single injection of estrogen into castrate female or intact male rats, the formation and subsequent regression of epithelial cords and cysts were observed to occur within 18 days (Glucksmann and Cherry, 1968). Cyst incidence was found to vary with the rat estrous cycle, being five to 10 times greater in estrus versus diestrus (Glucksmann and Cherry, 1968).

The thymus is part of the endocrine system; several well characterized peptide hormones are produced by the thymic epithelial cells (Monroe and Roth, 1986). To our knowledge, the thymic cyst epithelium has not been examined for the presence of thymic hormones. The purpose of this study was (1) to examine thymic cyst epithelium in adult dogs for the presence of the thymic hormones, thymosin alpha 1 and thymulin, and (2) to further characterize thymic cysts in adult dogs by light and electron microscopic examination, histochemical analysis of cyst contents, and immunocytochemical localization of keratin.

MATERIALS AND METHODS

Thymic tissues from 27 adult female beagle dogs of known age and medical history were utilized in this investigation. The dogs, all retired breeders purchased from Laboratory Research Enterprises (Kalamazoo, MI) and Marshall Farms (North Rose, NY) ranged in age from 33 to 84 months. Additionally, tissue for examination by transmission electron microscopy came from young adult, mixed breed dogs estimated to be 2 - 5 years old. Mixed breed dogs were obtained from the laboratory animal resources unit of the College of Veterinary Medicine, Iowa State University. All of the dogs had served as negative controls in other studies (Goff et al., 1987; Monroe et al., 1987). Dogs were euthanatized with sodium pentobarbital, and thymic tissue was collected immediately into 10% neutral buffered formalin or 3% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2.

Cyst morphology

Light microscopy For light microscopic examination, at least two thymic tissue samples per dog were processed routinely, embedded in paraffin, and sectioned at 4 - 6 μ m. Sample temperature was not allowed to exceed 60°C to facilitate antigen preservation for subsequent immunocytochemical analysis. Some sections from each subject were stained with hematoxylin and eosin; others were used for histochemical and immunocytochemical techniques as described later.

Scanning electron microscopy Samples of thymic tissue from four dogs were fixed in 10% neutral buffered formalin and subsequently placed into 3% glutaraldehyde in 0.1 M Sorenson's phosphate buffer, pH 7.2. The

tissue was then washed in 0.1 M cacodylate buffer, pH 7.2 and processed by the tannic acid method of Sweney and Shapiro (1977). Briefly, tissue was incubated in a solution of 3% glutaraldehyde/1% tannic acid in 0.1 M cacodylate buffer previous to dehydration in ethanol. Tissue to be cryofractured was taken from 100% ethanol and placed into liquid freon that was chilled over liquid nitrogen. When frozen, the tissue was placed on a metal block chilled with liquid nitrogen and was fractured with a razor blade. The samples were then placed in ascending concentrations of ethanol/freon solutions to pure freon, critical point dried, and sputter coated with gold-palladium. Samples were observed on a Cambridge Stereoscan 200 scanning electron microscope at 15 - 20 kV.

Transmission electron microscopy Thymic tissue was fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer, dehydrated in acetone, and embedded in Embed resin (Electron Microscopy Sciences, Ft. Washington, PA). Thin sections were stained with 2% methanolic uranyl acetate and Reynold's lead citrate, and observed on a Hitachi HS-9 electron microscope at 75 kv.

Histochemistry

The alcian blue (pH 2.5)/periodic acid Schiff's stain (Armed Forces Institute of Pathology (U. S.), 1960) for acidic and neutral mucins and the high iron diamine stain of Spicer (1965) for sulfated acidic mucins were used to investigate the nature of the cyst contents in comparison to previous reports.

Immunocytochemistry

Immunoreactive thymosin alpha 1, thymulin, and keratin were localized by the streptavidin-biotin immunoperoxidase technique (Biogenex, Dublin, CA). Diaminobenzidine tetrahydrochloride (DAB), DAB enhanced with nickel sulfate (Hancock, 1984), or 3-amino-9-ethylcarbazole (AEC) were utilized as chromogens. The antibody to thymosin alpha 1 was a gift from Dr. Paul Naylor, Alpha 1 Biomedicals (Washington, D.C.). Anti-keratin (directed to predominantly 56 and 64 kD keratin) was purchased from Dako Corporation (Santa Barbara, CA). Synthetic thymulin (Sigma Chemical Co., St. Louis, MO) was used to produce anti-thymulin according to the technique of Pleau et al. (1978). Briefly, synthetic thymulin was conjugated to bovine serum albumin (BSA, Sigma Chemical Co., St. Louis, MO) in the presence of glutaraldehyde. The thymulin-BSA solution was dialyzed against phosphate buffered saline, then injected intradermally, along with complete Freund's adjuvant, into New Zealand White rabbits. The rabbits were boosted monthly with thymulin-BSA conjugate and incomplete Freund's adjuvant; antiserum was collected bimonthly. Anti-thymulin antibody was separated from the whole serum by affinity chromatography using an activated CH Sepharose 4B column (Sigma Chemical Co., St. Louis, MO) according to Pleau et al., (1978). Enzyme-linked immunosorbent assays (ELISA's) were performed to determine the presence and titer of anti-thymulin antibody in the eluate from the affinity column.

RESULTS

Morphology

Light microscopy Thymic cysts were observed in 25 of the 27 subjects examined. The area of thymic tissue occupied by cysts varied between dogs, but seemed to correlate with age; the oldest dogs had the majority of thymic area occupied by cysts (Figure 1). There always remained at least a small remnant of thymic tissue identifiable by the gathering of lymphocytes and thymic epithelial cells, with occasional thymic corpuscles. The relationship of the thymic parenchyma to the cysts varied, depending on the amount of involution (lymphocyte loss) that had occurred. In slightly involuted thymus glands the cysts were completely surrounded by the parenchyma (Figure 1a), whereas in more severely involuted glands the cysts were surrounded by adipose tissue with only traces of lymphocytes nearby (Figure 1b). Small to medium sized arteries were usually found in the proximity of the cysts.

The cyst lining epithelium varied, sometimes even within one cyst, from squamous to cuboidal to columnar, with the predominant form being pseudostratified columnar epithelium with cilia (Figure 2). Aggregates of lymphocytes were sometimes observed directly beneath areas where the cyst epithelium was low and non-ciliated (Figure 2b).

The cyst lumina were either empty or contained an eosinophilic substance; cells, which appeared either viable or foamy and necrotic were sometimes observed within the lumina (Figure 3). In some regions the cyst epithelium seemed to end blindly within a thymic lobule, with the cyst lumen appearing continuous with the thymic parenchyma (Figure 4).

Figure 1. Light microscopic appearance of thymic cysts in a 3.5 year old (a) and a 7 year old (b) dog. c = cyst.
a, In slightly involuted thymus glands the cysts were mostly surrounded by lymphocytes of the thymic parenchyma. (bar = 200um)
b, In thymus glands that have undergone severe age-involution the cysts were surrounded by adipose tissue with only small groups of lymphocytes nearby. The arrows demarcate constricted regions between cyst chambers that may correspond to 'tunnels' such as those observed in a scanning electron microscopic preparation shown in Figure 5.

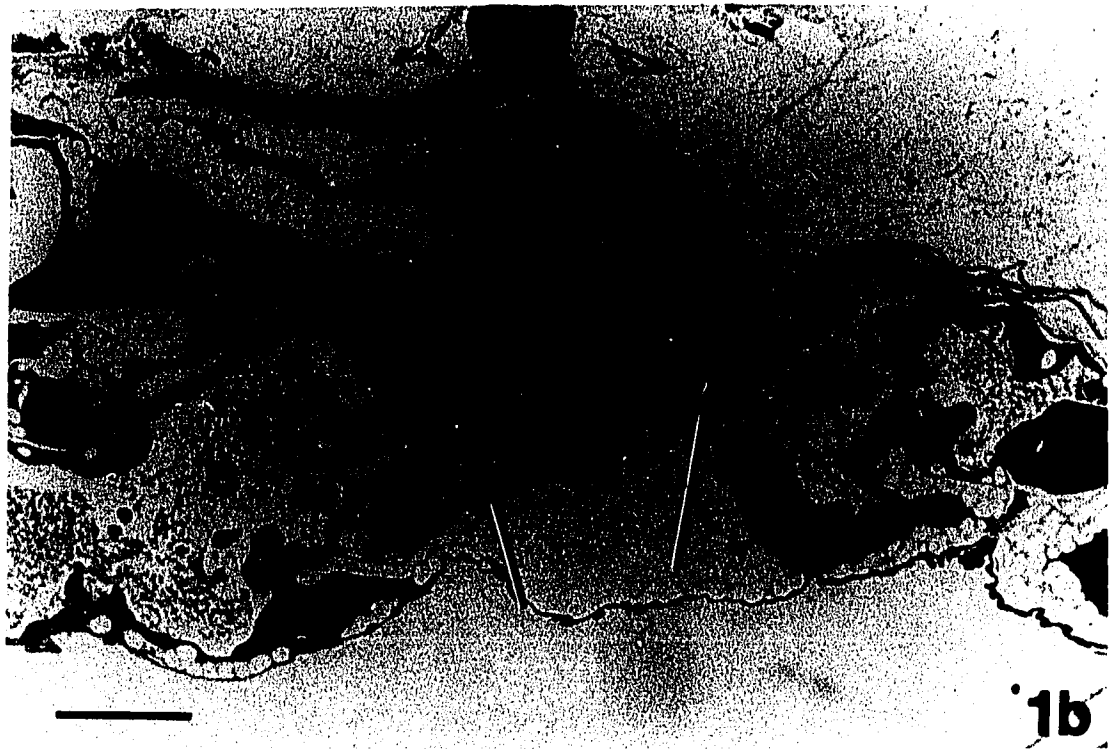
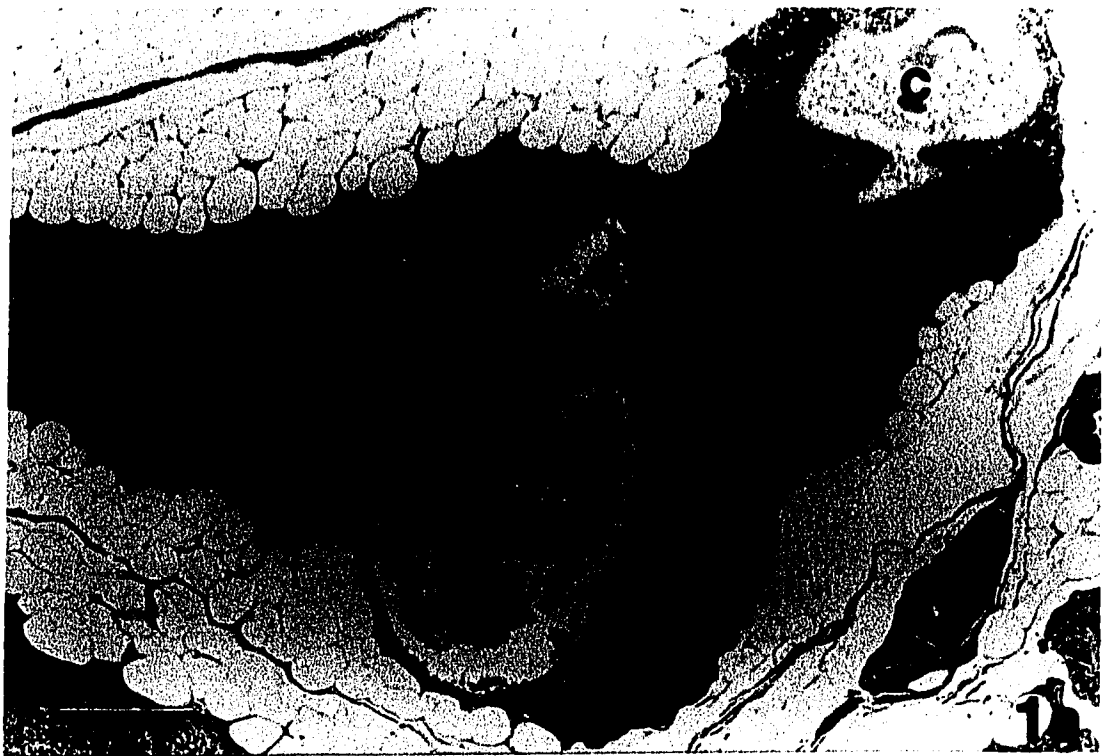


Figure 2. Light microscopic appearance of thymic cysts from a 4.5 year old dog.

a, The height of the cyst epithelium varied from squamous or low cuboidal to a pseudostratified columnar form; variations in height were observed even within a single cyst. (bar = 100um)

b, c, Some regions where the cyst epithelium was low and non-ciliated were associated with aggregates of lymphocytes (arrows). (bar = 100um)

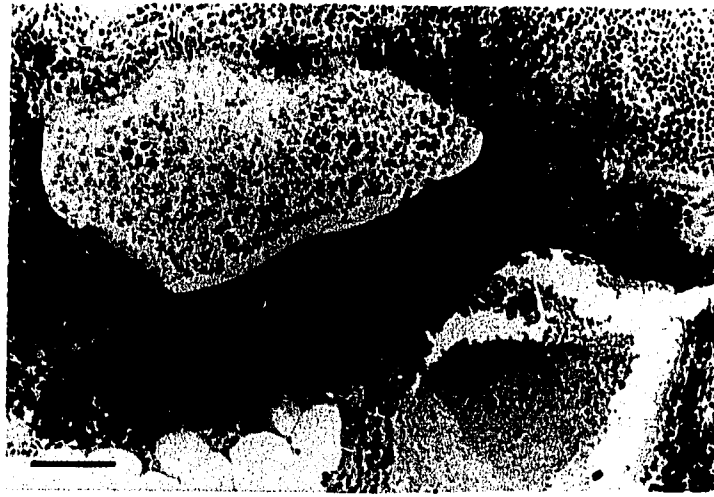


Figure 3. Light microscopic appearance of thymic cysts from a 5.5 year old dog. The cyst lumina sometimes contained cells with a necrotic (a) or viable (b) appearance, an eosinophilic, amorphous substance (c) or appeared empty (d). (bar = 100um)

Figure 4. Light microscopic appearance of a thymic cyst from a 5.5 year old dog showing the relationship between the cyst and the thymic lobule.

a, The cyst lumen appears to be continuous with the parenchyma of the thymic lobule. Brackets indicate the region which is enlarged in Figure 4b. (bar = 200um)

b, The cyst epithelium appears to end within the lobule (arrows); lymphocytes from the lobule (left) were observed within the cyst lumen (right). (bar = 100um)



Scanning electron microscopy The cyst lumen often contained a flocculent material embedded among cilia of the cyst epithelial cells (Figure 5). The epithelium resembled respiratory epithelium by being mostly ciliated, pseudostratified columnar; the folded apices of some cells bulged into the lumen (Figure 6). The epithelial layer as a whole was thrown into folds in some regions (Figure 6).

Transmission electron microscopy The cyst epithelium consisted of at least two cell populations that were separated from the thymic parenchyma proper by a basement membrane; these cells were the basal epithelial cells and the lumen lining cells (Figure 7).

The basal cells appeared rounded, with an irregularly shaped border due to multiple interdigitations with adjacent basal cells (Figure 7). Their nuclei were also irregularly shaped and contained moderately dense chromatin with some clumps of heterochromatin located peripherally, against the nuclear membrane. Basal cells were attached to the basement membrane by hemidesmosomes and to other basal cells by desmosomes. The moderately electron dense cytoplasm contained polysomes and mitochondria.

The lumen lining cells were predominantly columnar with cilia and microvilli protruding into the cyst lumen (Figures 7 - 8). The cilia appeared to have the typical 9 + 2 configuration of microtubules in cross section. The round to oval-shaped nuclei were located basally and contained one or two nucleoli along with sparse amounts of heterochromatin located against the nuclear membrane. Junctional complexes connected the cells at the apical region; elsewhere, occasional desmosomes connected adjacent cells. The cytoplasm contained tonofilaments and smaller fibrils, as well as microtubules. A small group of myoid elements was

Figure 5. a, Scanning electron micrograph showing the appearance of a cryofractured thymic cyst from a 4 year old dog. The arrows indicate 'tunnel' regions which may correspond to the constricted areas between cyst caverns shown in Figure 1. (bar = 500um)
b, Enlargement of central area from Figure 5a. Arrows point to 'tunnel' openings. (bar = 250um)

Figure 6. Scanning electron micrograph of cyst epithelium from the same preparation shown in Figure 5.
a, The predominant form of the cyst epithelium was pseudo-stratified columnar with cilia and microvilli. A flocculent material was sometimes observed embedded among the cilia. L = lumen. (bar = 25um)
b, The apical region of some cyst epithelial cells bulged into the cyst lumen and had a creased or folded appearance. (bar = 5um)
c, The cyst epithelium as a whole was thrown into folds in some regions. (bar = 50um)

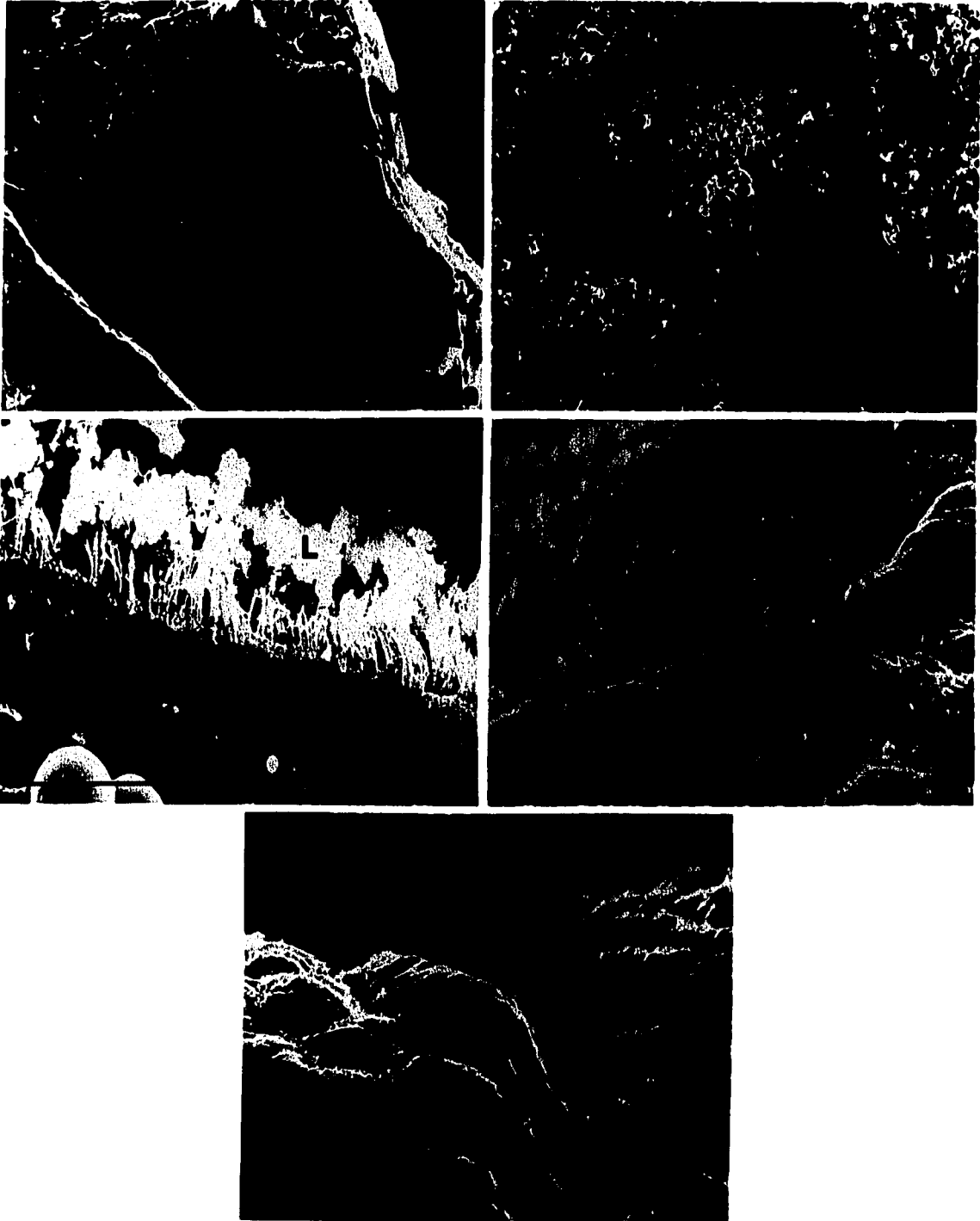
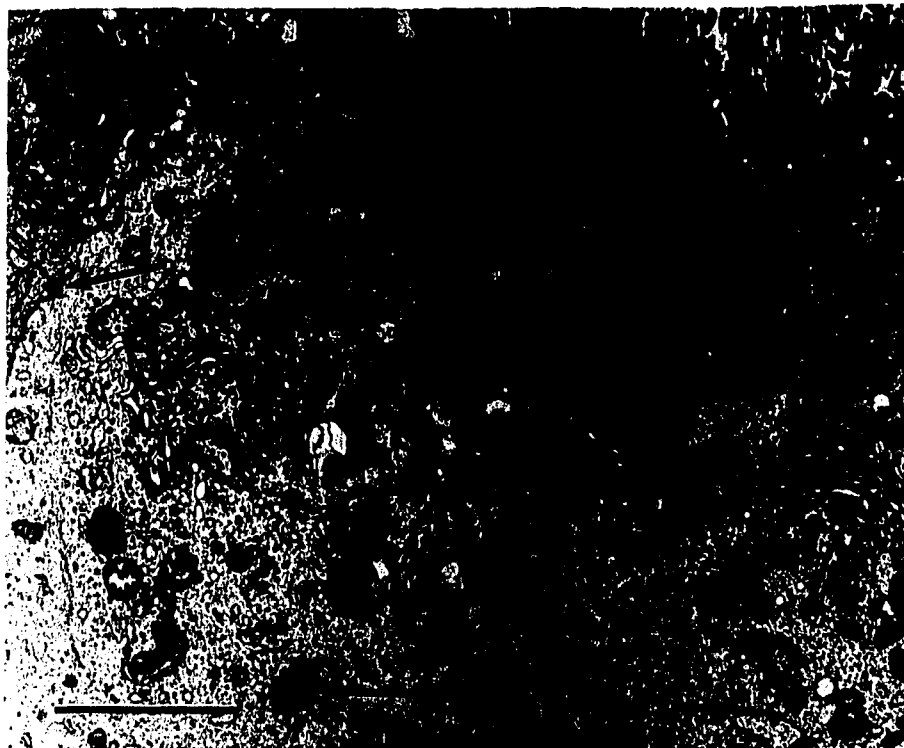
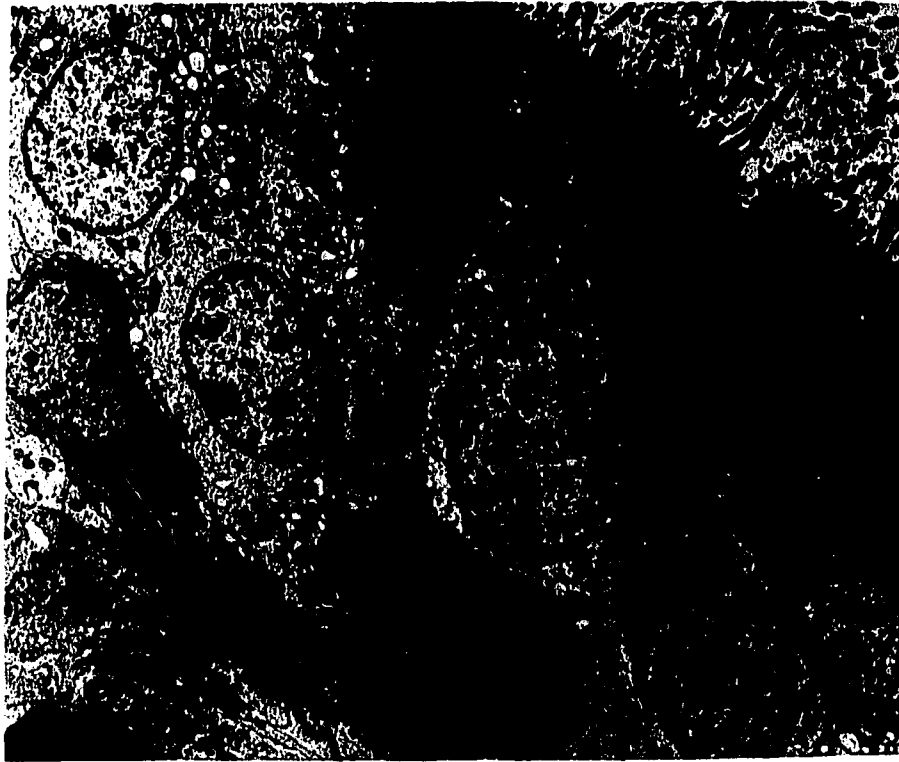


Figure 7. Transmission electron micrograph of cyst epithelial cells (apical region toward the top). b = basement membrane; bc = basal cell attached to basement membrane via hemidesmosomes; ec = epithelial cell. (bar = 1um)

Figure 8. Transmission electron micrograph of cyst epithelial cells (apical region toward the top). d = desmosome; g = granule; jc = junctional complex region; m = myoid element. (bar = 1um)



observed in one epithelial cell, the largest profile being 200 by 700 nm in diameter (Figure 9). Especially notable was an abundance of free polysomes; smooth and rough endoplasmic reticulum were present, but not abundant. In the apical region, one or two well developed Golgi complexes and many mitochondria (some of elongated form) were observed. Round to oval-shaped, membrane bound granules (250 - 500 nm in diameter) were also found in the apical region. Some granules had an eccentrically placed electron dense core which was either homogeneous or mottled in appearance.

Histochemistry

The contents of the cyst lumina and some cyst epithelial cells were stained turquoise and magenta with the alcian blue/PAS technique, indicating the presence of neutral and acidic mucins, respectively (Figure 10a). The grey to diffuse black color observed with the high iron diamine stain indicated the presence of sulfated acidic mucins, although the reaction was weak (Figure 10b).

Immunocytochemistry

Immunolabeled keratin was found in the majority of the epithelial cells lining cysts and in some, but not all, of the thymic epithelial cells present in the parenchymal tissue (Figure 11). In cyst epithelium, the keratin appeared mostly in the apical region (Figure 11). Some, but not all, of the cyst epithelial, parenchymal epithelial, and thymic corpuscle cells contained immunolabeled thymosin alpha one (Figure 12) or thymulin (Figure 13). The thymulin antiserum cross-reacted with the smooth muscle in blood vessels thus making 'background' staining appear high; however, the labelling in cyst epithelium is clearly specific as compared to control tissue (Figure 13).

Figure 9. Enlargement of transmission electron micrograph in Figure 8 showing the cell containing a myoid element (m). (bar = 500nm)

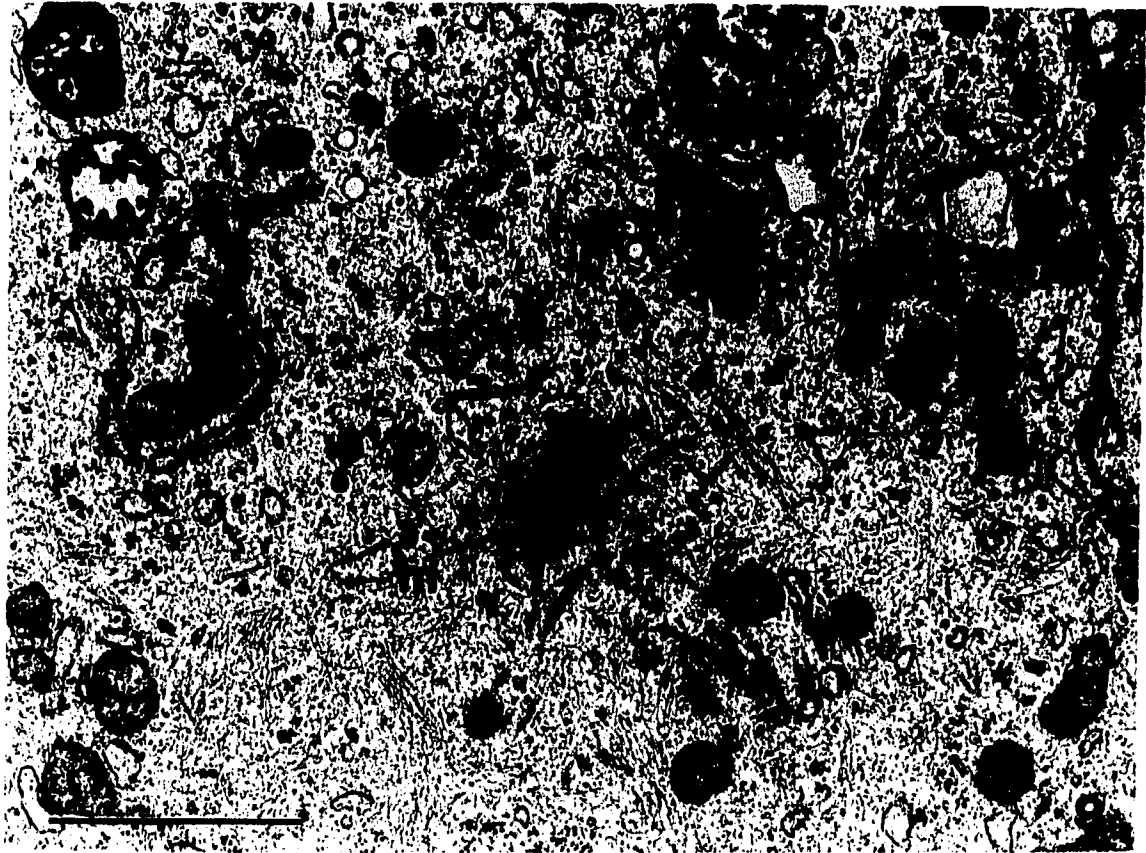


Figure 10. Thymic cyst from a 4 year old dog. L = lumen. (bar = 50um)
a, The alcian blue/periodic acid Schiff's stain resulted in the aqua and magenta staining of the contents of the cyst lumen, indicating the presence of neutral and acidic mucins.
b, The high iron diamine stain resulted in a gray to black staining of the contents of the cyst lumen indicating the presence of sulfated, acidic mucins

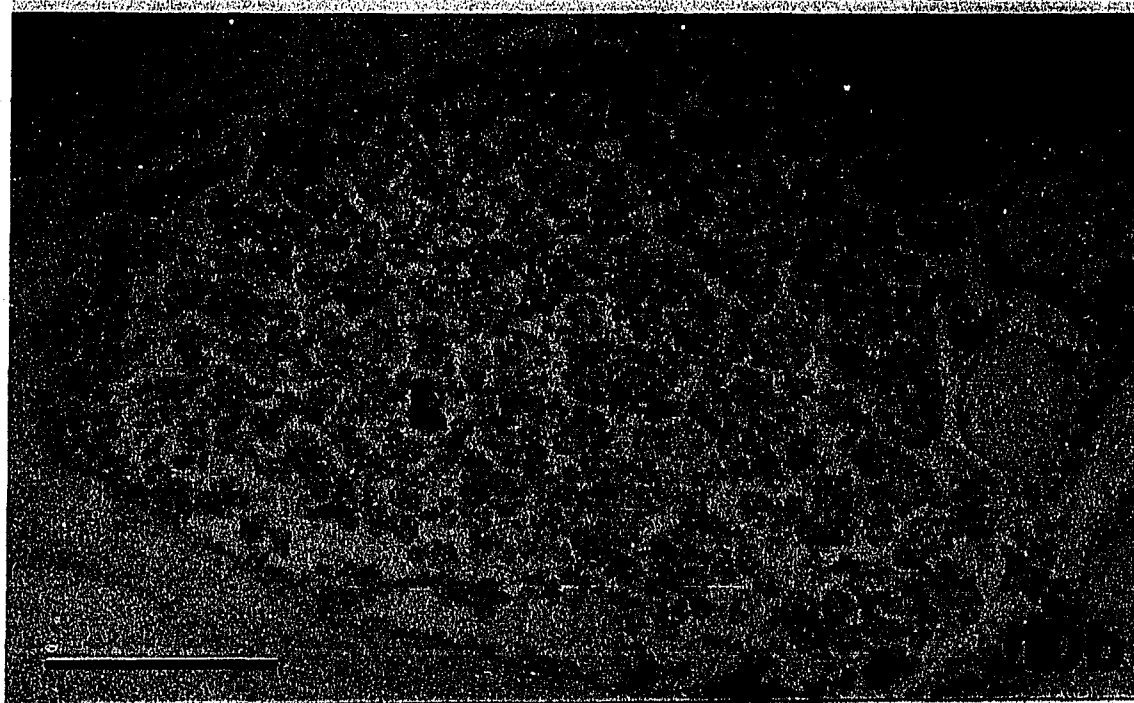
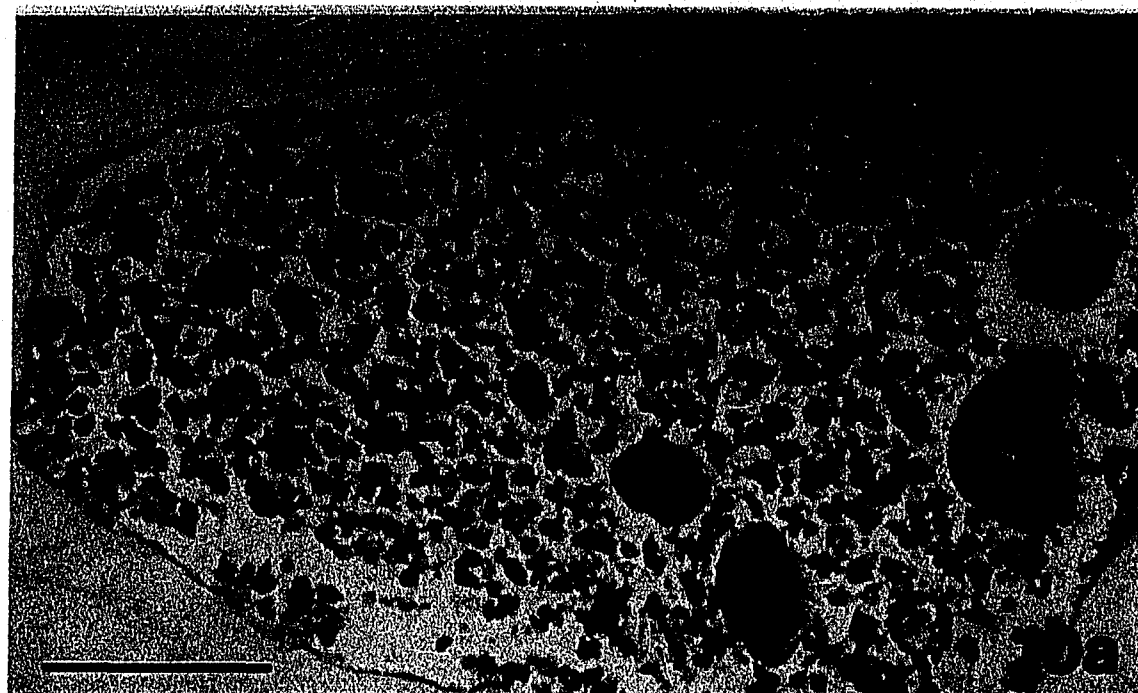
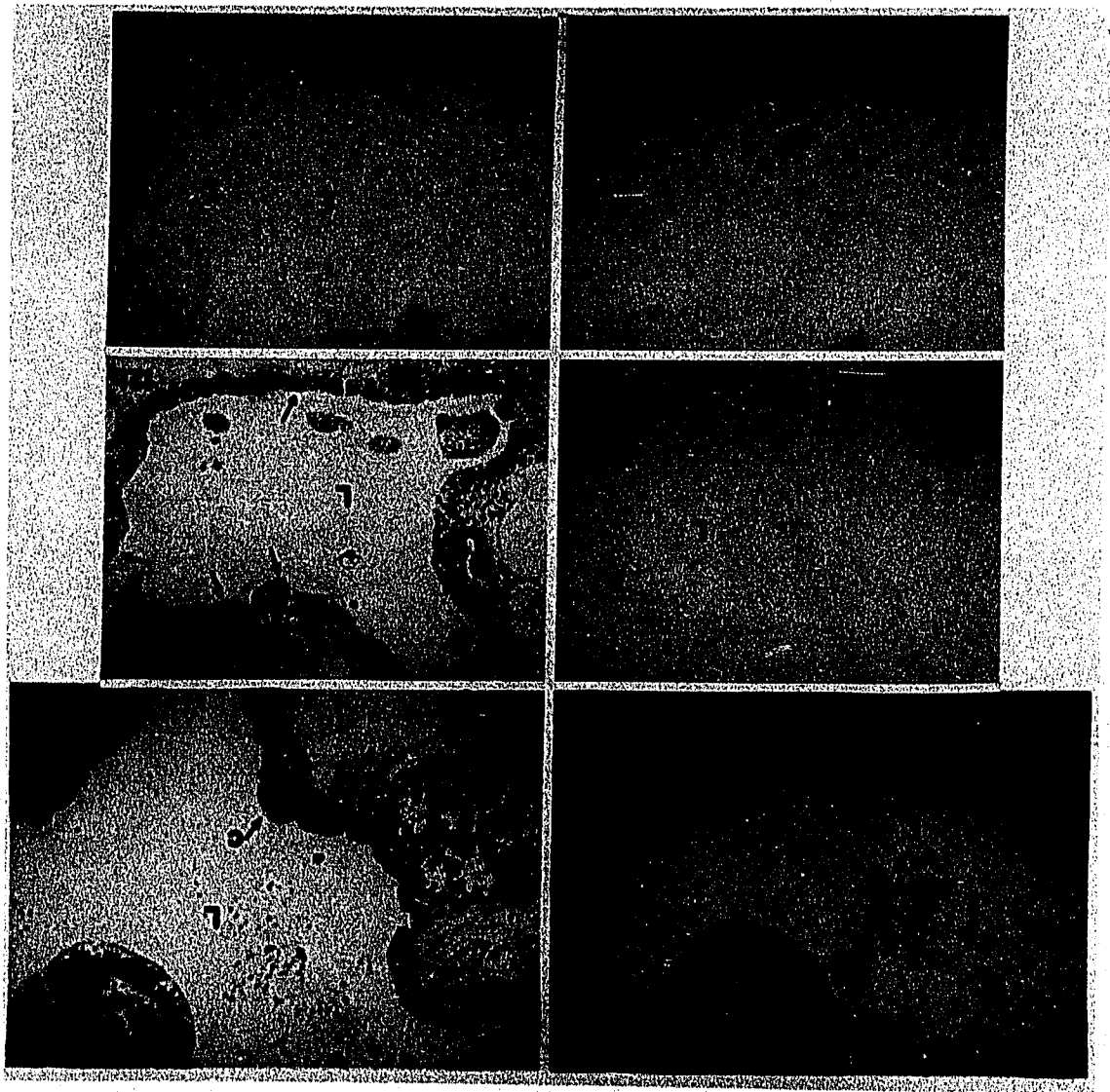


Figure 11. Immunocytochemical staining for keratin in a thymic cyst from a 6 year old dog. L = lumen. (bar = 100um)
a, Substitution control using non-immune rabbit serum.
b, Incubated with rabbit anti-human cytokeratin. AEC served as the chromogen resulting in a red reaction product where keratin was localized. c = cyst epithelium; e = thymic epithelial cell; t = thymic corpuscle

Figure 12. Immunocytochemical staining for thymosin alpha 1 in a thymic cyst from a 6 year old dog. L = lumen. (bar = 100um)
a, Substitution control using non-immune rabbit serum.
b, Incubated with rabbit anti-thymosin alpha 1. DAB enhanced with nickel sulfate served as the chromogen resulting in a purple-black reaction product where thymosin alpha 1 was localized in the tissue. Some immunolabelled cells are indicated by arrows

Figure 13. Immunocytochemical staining for thymulin in a thymic cyst from a 6 year old dog. L = lumen. (bar = 100um)
a, Substitution control using non-immune rabbit serum.
b, Incubated with rabbit anti-thymulin. DAB enhanced with nickel sulfate served as the chromogen resulting in a purple-black reaction product where thymulin was localized in the tissue. Some immunolabelled cells are indicated by arrows



DISCUSSION

The morphological observations of canine thymic cysts at the light microscopic level in this study agree with those previously reported (Oksanen, 1968). With increasing age the incidence of cysts increases and they are surrounded by less parenchymal tissue and more adipose tissue (Figure 1). The epithelium, which varies in height, surrounds a lumen that may be empty or filled with an eosinophilic amorphous mass (Figures 2 - 3). In some regions it appeared that the cyst lumen was continuous with or opened into the thymic parenchyma; in those regions especially, lymphocytes could be observed in the cyst lumen (Figures 3 - 4). Others have reported observing lymphocytes within thymic cyst lumina, or crossing the cyst epithelium; one hypothesis is that the cyst lumen may serve as a pathway between the cortex and medulla during lymphocyte differentiation (Nabarra and Andrianarison, 1987; Meihuizen and Burek, 1978).

Some investigators consider thymic cysts to be remnants of the branchial epithelium from which the thymus develops (Newman, 1971; Liu et al., 1983); the term 'remnant' carries the connotation of the structure being inactive. The high incidence of thymic cysts in adult dogs, and their paucity in normal young dogs, argues against the interpretation of these structures as branchial remnants (Oksanen, 1968; Watney, 1882; White, 1942; Newman, 1971; Bodey et al., 1987). Other authors have interpreted thymic cysts as derivatives of degenerating thymic corpuscles (Watney, 1882; Hammar, 1921). We prefer to reserve the term thymic (or Hassall's) corpuscles for groups of concentrically arranged, degenerating, keratinized epithelial cells; thymic corpuscles are easily distinguished

from thymic cysts. Even Watney (1882) observed the active state of the cyst epithelium and noted that this would not be expected of degenerating cells.

As noted by Oksanen (1968), White (1942), and Newman (1971) the incidence of thymic cysts appears to be related to increasing age. This positive correlation between cyst incidence and age has been an incidental finding in our previous examinations of canine thymus glands from dogs of various ages, and is noted again here. We must not disregard the possibility of missing the presence of small cysts in younger dogs due to sampling error. It is possible that cords of epithelial cells present even in the thymus of young animals may correspond to cysts that will become more apparent to the observer in the uncrowded environment of the involuted thymus. If this were the case, then the incidence of cysts would only appear to increase with age due to increases in the size and content of the cysts with age.

The positive correlation with age seems to negate the notion put forth by many authors dismissing thymic cysts as remnants of the branchial epithelium. If these structures were true branchial remnants we should expect the incidence of cysts to be equal in the young and the aged and the frequency of occurrence to be much lower than that found in this and other studies (Oksanen, 1968; Watney, 1882; White, 1942; Newman, 1971). Oksanen (1968) reported that the greatest correlation was observed between cyst incidence and the degree of accidental or acute involution.

To our knowledge no previous report of the scanning electron microscopic (SEM) appearance of thymic cysts has been published. In the three dimensional SEM view of the cyst regions the lumina appeared as

chambers containing a flocculent substance (Figure 5). The opening of what appeared to be a tunnel between two cyst chambers (Figure 5) probably corresponds to a similar region observed at the light microscopic level (Figure 1). The cyst epithelium was thrown into folds in some regions; the folded apices of some cells bulged into the lumen (Figure 6). The cross sections of cysts seen at the light microscopic level probably represent regions of one or a few connected cysts, which are somewhat convoluted in form.

The appearance of canine thymic cysts at the transmission electron microscopic level has not been previously reported. The present observations of the ultrastructure of the cyst epithelium (Figures 7 - 8) agree with those reported for the rat (Meihuizen and Burek, 1978) and human (Vetters and Macadam, 1973). Those authors concluded that the cyst epithelium has the cellular machinery required for protein synthesis and secretion (Meihuizen and Burek, 1978; Vetters and Macadam, 1973). The granules observed in the apical regions have been hypothesized to be secretory granules; ^3H -leucine incorporation studies suggested that polypeptide synthesis and secretion were occurring in thymic cysts (Meihuizen and Burek, 1978). Another interpretation of the micrographs are that the granules are phagolysosomes. Techniques to localize lysosomal enzymes at the TEM level might clarify this question. It is possible that both of these interpretations are correct, for lysosomes in secretory cells sometimes function in the recycling of secretory granules, forming phagolysosomes with an appearance very much like the granules observed in the cyst epithelium. Since seasonal and hormonal influences on the cysts may change the secretory activity of the epithelium, the

samples observed in this study may have been collected at a time of decreased activity. A comparison of canine cyst epithelium at various seasons, and during different stages of the estrous cycle would be of interest.

The presence of a myoid element in a cyst epithelial cell (Figure 9) is not too surprising since some of the epithelial cells of the thymic parenchyma contain myoid elements and have even been implicated as an antigen source in the formation of 'anti-muscle' antibodies (actually anti-acetylcholine receptor) in myasthenia gravis (Kendall, 1981). It is interesting that Kendall (1981) noted not only a seasonal change in cyst incidence, but also in myoid cell incidence. As the present results indicate, the cyst epithelia and myoid cells may be derived from the same cell type and/or are under similar controls.

The present results concur with those of Oksanen (1968) and Newman (1971) who reported that the cyst epithelium produces neutral and acidic, sulfated mucins (Figure 10). The role of such a secretion in the thymus is unclear. Other body regions where mucous membranes are found include the respiratory, gastrointestinal, and reproductive tracts. In these regions, which eventually open to the external environment, the mucus is thought to serve a protective function, and is carried along by the beat of the cilia. It is not known if the cilia in thymic cysts beat, or where the secretion would go to if they did. An interesting analogy of the thymic cysts to thyroid follicles was made by Meihuizen and Burek (1978); the cyst epithelium may be secreting into and reabsorbing from the cyst lumen. Other investigators have noted this resemblance to thyroid follicles; the thymic cysts do not incorporate radioactive iodine (Clark,

1963). It would be of interest to know if the thymic cysts are indeed storing a secretory product in the lumina.

Immunolabeled thymosin alpha 1 and thymulin were detected in some cyst epithelial cells (Figures 11 - 13). This is the first direct evidence of thymic hormone production by cyst epithelia that we are aware of. This certainly lends credence to the hypothesized secretory function of thymic cysts. Since only some of the epithelial cells were immunolabeled for thymic hormone, it would be of interest to attempt to stimulate the secretion of the cysts (for example with hormonal treatment or by sampling at a particular period of the estrous cycle) and then examine the epithelium for the presence of thymic hormone.

As previously discussed, the thymic cysts resemble mucous membranes found elsewhere in the body. In many body regions specialized cells of the mucous membrane are associated with aggregates of lymphoid tissue, and produce secretory component related to IgA produced by local plasma cells; collectively these regions are known as the mucosal associated lymphoid tissue or MALT. It is interesting to note, in this regard, those regions where lymphocytes are gathered beneath non-ciliated, flattened areas of the thymic cyst epithelium (Figure 2b); these are somewhat reminiscent of dome regions in the other MALT regions. The production of secretory component by human thymic epithelial cells has been reported (Tomasi and Yurchak, 1972). These authors proposed that secretory component in the thymus may influence differentiation and/or migration of a population of T-lymphocytes towards IgA producing cells and secretory (mucous membrane) sites. Further work is needed regarding the prevalence of these dome-like

regions and their relationship, if any, to those cells producing secretory component.

SUMMARY AND CONCLUSIONS

Section I of this study presented a review of the literature on thymotrophic agents and their influence on immune function. The overall purpose of this study was to further characterize thymic structure and immune function in aging dogs.

In a previous study, growth hormone (GH) was observed to be thymotrophic in a diverse group of aging dogs (Monroe et al., 1987). The study presented in Section II was undertaken to determine if GH would improve thymic morphology and endocrine function in a better defined group of dogs. The administration of GH was associated with an increase in thymulin (a thymic hormone) production in every dog regardless of its effect on thymic morphology. These results indicate that the function of the thymic epithelial cells may be affected by exogenous treatments without a concurrent change in overall thymic morphology. This denotes a problem of assuming changes in thymic function based on simple thymic morphology or weight, as many authors have done.

One problem with using exogenous hormones as thymotrophic factors is the possibility that the subject will begin producing antibodies to the foreign protein, thereby inactivating it. In a previous study (Morrison, 1987) this potential problem was overcome by using clonidine HCl to induce endogenous GH secretion in aging dogs. Some enhancement of thymic morphology and immune function was noted; however, the dogs became tolerant to the daily administration of the drug (Morrison, 1987). As presented in Section III, intermittent administration of clonidine HCl may have overcome the problem of desensitization to the drug; this regimen was

associated with enhancement of the primary antibody response to B. abortus. There was a problem with the formulation of the clonidine; the enteric coated tablets seemed to have delayed absorption of the drug resulting in delayed and inconsistent GH responses. For future studies, a liquid formulation of clonidine is recommended. Based on the results of the present and previous studies, further investigation of clonidine as an immunoenhancing agent is warranted.

It was not long ago that the immune and endocrine functions of the thymus were recognized. There is still much to learn about this dynamic organ. As presented in section IV, the exact significance of thymic cysts is still not known, although their anatomy was first described over 100 years ago (Watney, 1882). The present results demonstrate the ultrastructural similarities between cyst epithelium in the dog and other species. Cyst incidence increases with age or precocious involution and is also affected by certain endocrine manipulations. Mucous secretion by thymic cysts has been frequently reported; hormone secretion has been repeatedly hypothesized without direct evidence. In this study, the presence of at least two thymic hormones was demonstrated immunocytochemically. Further investigation of the epithelial cell granules and of the cyst lumen contents may clarify the function of these structures.

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ACKNOWLEDGMENTS

Without my family, friends, and my faith in God I don't think I could have ever completed this work. My mother and my late father, Betty and Elan Lawler, have always encouraged me in the pursuit of my education; their sense of pride in me has kept me going these many years. My children, Marilee and Sean, have been tolerant of my absence during the writing of this dissertation (or at least as tolerant as a 4 year old and a 2 year old can be when it's time to go visit the Easter bunny at the mall). Jesse, my husband, has been a source of great moral support, valuable editorial comments on the manuscript, and a terrific babysitter!

I would like to thank my major professor, Dr. Jim Roth, for his guidance, support, and friendship. To the other members of my committee, Dr. Lawrence Arp, Dr. Jeanine Carithers, Dr. Merlin Kaeberle, and Dr. Etsuro Uemura, my thanks for your willingness to discuss various aspects of my research and to share your expertise with me.

A special thank you to Miss Andrea Dorn for her technical support, laboratory expertise, and friendship. Finally, I thank those friends and fellow graduate students who have kept me going these last few months by their kind words and deeds.

This study was supported by PHS Grant numbers AG05517 (to J. Roth) and AG02814 (to G. Incefy) awarded by the National Institute on Ageing, and CA33168 (to G. Incefy) awarded by the National Cancer Institute, DHHS; and a grant from Ralston Purina, Inc., St. Louis, MO (to J. Roth).

This research was conducted according to the rules and regulations of the Animal Welfare Act.

